UNIVERSITY OF ALBERTA

IMMUNE MODULATION THERAPY FOR THE PREVENTION OF AUTOIMMUNE DIABETES AND INDUCTION OF TOLERANCE IN EXPERIMENTAL ISLET TRANSPLANTATION

BY

WAYNE TRUONG ©

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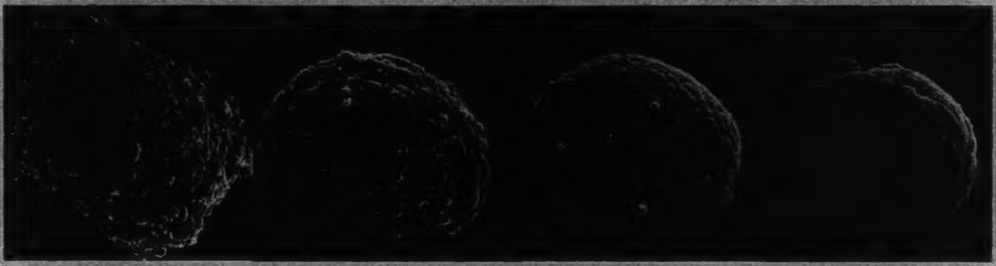
Wayne

Dr. Wayne Truong

3 October 2007

"An obstacle is something you see when you take your eyes off the goal."



"History tells us that procedures that were inconceivable yesterday, and are barely achievable today, often become the routine of tomorrow."

Dr. Thomas E. Starzl

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **IMMUNE MODULATION THERAPY FOR THE PREVENTION OF AUTOIMMUNE DIABETES AND INDUCTION OF TOLERANCE IN EXPERIMENTAL ISLET TRANSPLANTATION** submitted by **WAYNE TRUONG** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY IN EXPERIMENTAL SURGERY**.

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31 August 2007

DEDICATION

This thesis is dedicated to my love, Jenny, who has inspired me with endless enthusiasm, passion, and imagination, and has brought me balance and strength throughout my journey, and has taught me to cherish every step on the road less traveled by, and to my family whose love, encouragement, and support are boundless.

ABSTRACT

Islet transplantation offers improved glycemic control for patients with severe, uncontrolled type 1 diabetes, but currently relies on life-long immunosuppression to prevent both allograft rejection and recurrent autoimmunity. Developing immunomodulatory strategies to minimize drug toxicity, and ideally to induce tolerance are major goals to expand the indications for islet transplantation for treatment of autoimmune diabetes.

Although strategies to prevent acute allograft rejection in experimental murine models are common, clinical transplantation tolerance induction remains elusive. Development of therapies that prevent auto- and alloimmunity are of the highest priority in islet transplantation. Two promising strategies include modulation of lymphocyte trafficking and selective targeting using monoclonal antibodies (mAb) specific for novel coinhibitory receptors. FTY720 and B and T lymphocyte attenuator (BTLA), may complement the natural mechanisms that limit lymphocyte activation and function. The overall goal of this work is to develop tolerance-inducing immune modulating therapies in experimental murine models of autoimmune diabetes and islet transplantation, with potential implementation in the clinic.

Chronic administration of FTY720 binds and down-regulates the sphingosine-1-phosphate receptor (S1PR) surface expression and causes sequestration of lymphocytes within lymphoid tissue thereby preventing cell-mediated damage to target tissue. Although islets also express S1PR, no negative impact of FTY720 exposure was found when human islets were evaluated: (i) *in vitro* using glucose-stimulated insulin and apoptosis assays; and, (ii) *in vivo* after transplantation into immunodeficient mice by monitoring blood glucose, oral glucose tolerance, and human C-peptide secretion.

Emerging S1PR modulators should also be investigated for potential islet toxicity before clinical application.

Selectively targeting auto- and alloaggressive BTLA expressing lymphocytes using a depleting BTLA-specific mAb (6F7) significantly delayed the onset of diabetes in non-obese diabetic (NOD) mice, and induce long-term fully MHC-mismatched islet allograft survival with evidence of donor-specific tolerance when combined with CTLA4Ig costimulatory blockade and accumulation of Foxp3+ lymphocytes within the graft. Furthermore, a non-depleting anti-BTLA mAb (PJ196) paradoxically enhanced islet allograft survival in the context of CTLA4Ig costimulatory blockade with BTLA expression downregulation.

The experimental results from this thesis indicate that cell trafficking and coinhibitory modulation may be effective adjunctive tolerance-inducing strategies for rational application in clinical islet transplantation.

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
AUC	area under the curve
BCR	B cell receptor
BMT	bone marrow transplant
BTLA	B and T lymphocyte attenuator
CD40L	CD40 ligand
CFSE	carboxyfluorescein diacetate succinimidyl ester
CNI	calcineurin inhibitor
CsA	cyclosporine
CTLA4	cytotoxic T lymphocyte associated antigen-4
DST	donor specific transfusion
GVDH	graft-versus-host disease
HbA_{1c}	hemoglobin A_{1c}
HSC	hematopoietic stem cells
HVEM	herpesvirus entry mediator
IBMIR	instant blood mediated inflammatory reaction
ICOS	inducible costimulatory molecule
Ig	immunoglobulin
IL	interleukin
mAb	monoclonal antibody
MHC	major histocompatibility complex
NHP	non-human primates
NOD	non-obese diabetic
PD-1	programmed cell death-1
PERV	porcine endogenous retrovirus
PFC	perfluorocarbons
S1P	sphingosine-1-phosphate
S1PR	sphingosine-1-phosphate receptor
TCR	T cell receptor
Tim	T cell immunoglobulin mucin
Tregs	regulatory T cells

PREFACE

Cellular and solid organ transplantation still depends on chronic immunosuppression to prevent allograft rejection, which increases the risks of malignancy, opportunistic infection, and drug toxicity. Islet transplantation faces the additional challenge of recurrent autoimmune disease. The ultimate goal of tolerance may be required to liberate patients from chronic immunosuppression and recurrent disease. The most promising experimental strategies to induce tolerance include targeting T cell trafficking receptors and recently identified positive and negative co-signaling molecules. The development of novel agents that address both allograft rejection and autoimmune recurrence are paramount for improving islet transplantation outcomes.

The **overall goal** of this thesis is **to develop tolerance-inducing immune modulating therapies in murine models of autoimmune diabetes and islet transplantation**, with potential for rational clinical application. The impact of the cell trafficking regulatory molecule, FTY720, on human islet function and the potential role of coinhibitory receptor B and T lymphocyte attenuator (BTLA) in regulating auto- and alloimmunity, has not previously been fully investigated. The **major aims of this thesis** include: (i) evaluating β cell toxicity and the impact of FTY720 exposure on human islets, as **a model for testing new drugs for islet transplantation**; and, (ii) investigating **the role of BTLA coinhibitory pathway in autoimmune diabetes and islet allograft rejection and tolerance**.

CHAPTER ONE

PROGRESS IN ISLET TRANSPLANTATION FOR PATIENTS WITH TYPE 1 DIABETES MELLITUS

NOTE: This chapter has been extensively updated and modified from
a previous version of an article published in
Treatments in Endocrinology 2006; 5(3): 147-58.
The authors on this paper include: Truong W, Shapiro AMJ.

1.1 OVERVIEW

More than 500 patients with T1DM have now received islet transplants at over 50 institutions worldwide in the past 5 years. Insulin independence rates with current protocols are impressive at one year. However, inexorable decay of islet function over time indicates that there are many opportunities for improvement. Improved control of glycosylated hemoglobin (HbA_{1c}) and reduced risk of recurrent hypoglycemia are seen as important benefits of islet transplantation irrespective of the status of insulin independence. For islet transplantation to expand it is essential that the donor to recipient ratio be reliably reduced to 1:1. Enormous opportunities lie ahead for the development of successful living donor islet transplantation, single donor protocols, improved engraftment, islet proliferation *in vitro* and in the recipient, and novel tolerizing drugs. With these emerging opportunities, islet transplantation may expand to include more patients with type 1 diabetes including children, and will not be restricted to the most unstable forms of the disease as it is today.

1.2 INTRODUCTION

Diabetes mellitus is a common endocrine disorder affecting over 200 million people worldwide, representing 6% of the population, and is the fourth leading cause of death in North America. Although the majority of adult patients with diabetes have type 2 disease, type 1 is the most severe form resulting from selective and progressive autoimmune destruction of insulin-producing β-cells within the islets of Langerhans in the pancreas. The loss of β-cell function leads to insulin insufficiency and uncontrolled hyperglycemia.

The discovery of insulin significantly altered the fate of patients with type 1 diabetes by preventing acute lethal complications like diabetic ketoacidosis (*1*). Improved patient survival, however, allowed the development of secondary complications of diabetes including atherosclerosis, lipid disorders, proliferative retinopathy, peripheral neuropathy, and renal failure. Evidence illustrating the importance of maintaining strict glycemic control was established in 1993 with the landmark Diabetes Control and Complications Trial (DCCT) study (*2*). With intensive insulin therapy, the glycosylated hemoglobin (HbA_{1c}) was 1% lower than the control group and significantly protected against microvascular complications, nephropathy, neuropathy, and retinopathy. Similarly, the United Kingdom Prospective Diabetes Study (UKPDS) showed a significant 1% decrease in the HbA_{1c} of patients with type 2 diabetes with microvascular disease (*3*). However, the cost of improved glycemic control was associated with a threefold increase in serious hypoglycemic events, including recurrent seizures and coma (*4*).

Consistent glycemic control using exogenous insulin remains a challenge. Administration of insulin by subcutaneous injection is non-physiologic and inherently limited by delayed absorption, variable blood levels, and systemic rather than portal venous delivery. Even with newer insulin analogues combined with intensive therapy, which has removed some of the delay in absorption and variability, normoglycemia is difficult to achieve. Continuous subcutaneous insulin delivery, attention to diet, and carbohydrate counting improve, but do not completely normalize blood glucose levels. β cell replacement by whole pancreas or islet transplantation can physiologically achieve this goal.

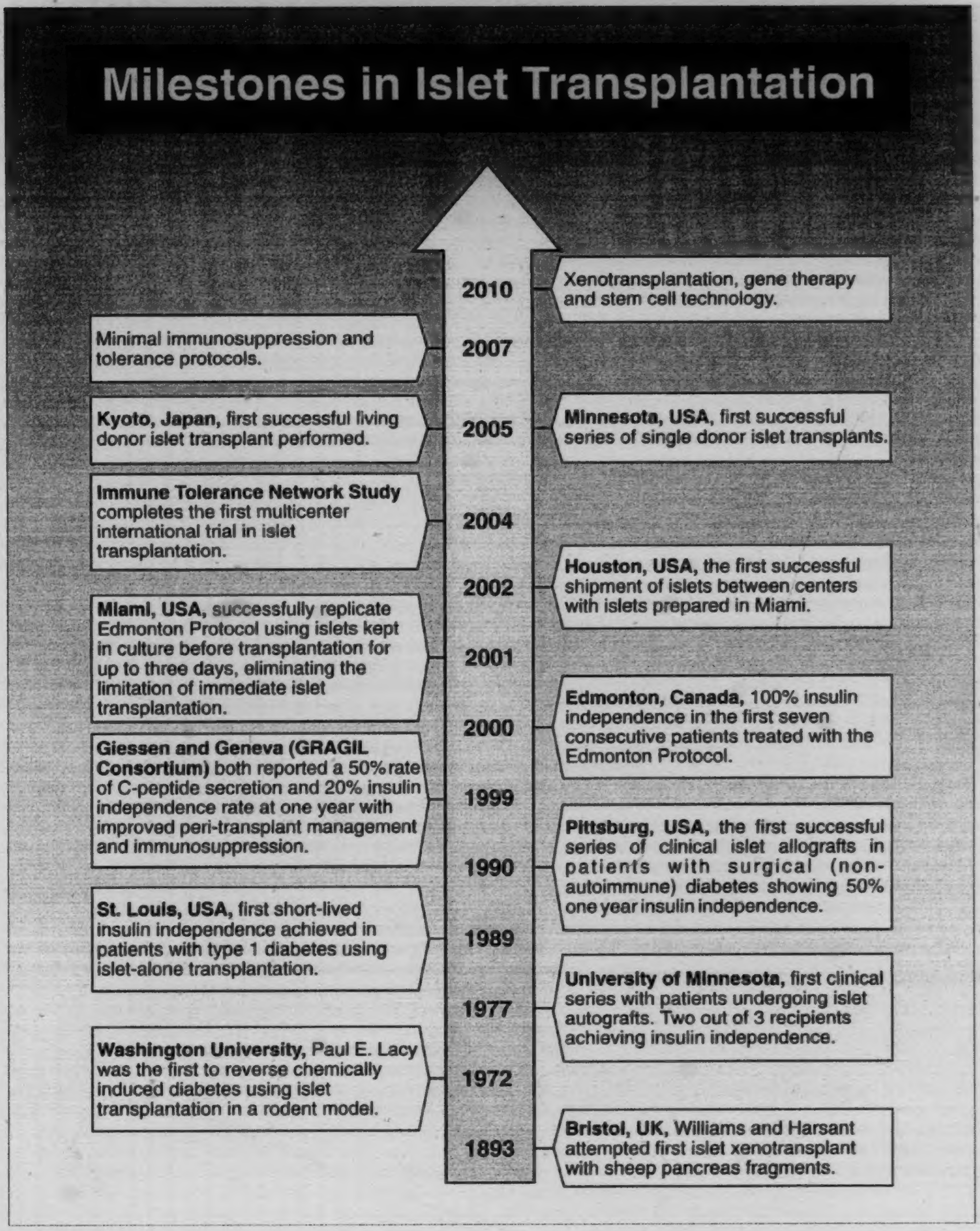
Whole pancreas transplantation can prolong life, reverse established nephropathy (*5*), and improve quality of life, but it remains too invasive for the vast majority of patients. Over 21,000 whole pancreas transplants have now been performed worldwide, and recent data from the major centers report remarkable improvement in outcome, with one year pancreas graft survival exceeding 85%, and patient survival exceeding 90%. The use of portal venous drainage, enteric exocrine drainage and use of steroid sparing, more potent immunosuppression strategies have also contributed to these improved outcomes.

Islet transplantation compared to whole pancreas transplants has the advantage of fewer complications and the elimination of unnecessary transplantation of the exocrine component of the gland. The Edmonton report of seven consecutive successful human islet transplants, in the year 2000, has renewed optimism in β-cell replacement as a potential cure for type 1 diabetes mellitus (*6*).

Despite phenomenal progress in the field of islet transplantation, many challenges and emerging opportunities still remain. Here we present a summary of the origin, evolution, current limitations, and future direction in islet transplantation for the treatment of T1DM.

1.3 THE ORIGIN OF ISLET TRANSPLANTATION

Investigators have been exploring the possibility of treating diabetes mellitus with pancreatic replacement strategies well before the discovery of insulin (FIGURE 1-1). In 1892 Minkowski and von Merring noted that diabetes associated with total pancreatectomy in canine subjects could be prevented by replacing a portion of the pancreas into the subcutaneous tissue (7). Soon after this report, Watson-Williams and his surgical colleague Harsant, of the Bristol Royal Infirmary in the UK, attempted the first clinical islet (pancreas) transplantation when they placed three pieces of freshly slaughtered sheep's pancreas subcutaneously into a fifteen-year-old boy with suspected ketoacidosis in 1893 (8). Without knowledge of immunologic barriers at the time, however, the xenograft was destined to fail. Attention turned away from islet transplantation with the discovery of insulin by Banting, Best, Collip and Macleod in 1922 (9). By the following year, clinical administration of exogenous insulin had transformed type 1 diabetes mellitus (T1DM) from a rapidly fatal disorder, after the onset of ketoacidosis, to a chronic incurable disease with secondary complications. Furthermore, vascularized whole pancreas transplantation emerged when Kelly and Lillehei, at the University of Minnesota, performed the first simultaneous kidney-pancreas transplant in 1966 (10). It was not until 1967 with the development of collagenase digestion of the pancreas (11) that islet transplantation was again considered a potential treatment for patients with T1DM. Paul Lacy, at Washington University in St. Louis in 1972, was the first to successfully reverse chemical diabetes in rodents through the transplantation of isolated islets of Langerhans (12). Replacement of the β cells that are selectively destroyed in T1DM appeared to be conceptually superior and much safer than whole pancreas transplantation (13).



2010 Xenotransplantation, gene therapy and stem cell technology.

2007 Minimal immunosuppression and tolerance protocols.

2005 **Kyoto, Japan,** first successful living donor islet transplant performed.

2005 **Minnesota, USA**, first successful series of single donor islet transplants.

2004 **Immune Tolerance Network Study** completes the first multicenter international trial in islet transplantation.

2002 **Houston, USA,** the first successful shipment of islets between centers with islets prepared in Miami.

2001 **Miami, USA,** successfully replicate Edmonton Protocol using islets kept in culture before transplantation for up to three days, eliminating the limitation of immediate islet transplantation.

2000 **Edmonton, Canada,** 100% insulin independence in the first seven consecutive patients treated with the Edmonton Protocol.

1999 **Giessen and Geneva (GRAGIL Consortium)** both reported a 50% rate of C-peptide secretion and 20% insulin independence rate at one year with improved peri-transplant management and immunosuppression.

1990 **Pittsburg, USA,** the first successful series of clinical islet allografts in patients with surgical (non-autoimmune) diabetes showing 50% one year insulin independence.

1989 **St. Louis, USA,** first short-lived insulin independence achieved in patients with type 1 diabetes using islet-alone transplantation.

1977 **University of Minnesota,** first clinical series with patients undergoing islet autografts. Two out of 3 recipients achieving insulin independence.

1972 **Washington University,** Paul E. Lacy was the first to reverse chemically induced diabetes using islet transplantation in a rodent model.

1893 **Bristol, UK,** Williams and Harsant attempted first islet xenotransplant with sheep pancreas fragments.

Figure 1-1 Milestones in Islet Transplantation: Past, Present, and Future

1.4 THE EVOLUTION OF CLINICAL ISLET TRANSPLANTATION

Over the subsequent years, the transition from bench to bedside proved to be difficult and frustrating. Centers were performing clinical allo- and auto- islet transplantation in the 1970s (14-16). Najarian and colleagues, at the University of Minnesota, were the first to complete a clinical series of islet transplants for patients with T1DM in 1977. Seven patients with previous renal allografts received islet allotransplants, but none achieved insulin independence (17). They went on to conduct another small series with patients who underwent intraportal islet autografts after sub-total pancreatectomy for chronic pancreatitis. Two out of the three patients achieved insulin independence for more than a year, showing the clinical usefulness of the technique proposed by Lacy (18). However, many obstacles remained and this series did not address the issues of alloimmunity or autoimmunity (associated with T1DM). A group in Zurich, Switzerland reported a series of patients with T1DM receiving islet transplantation delivered through the splenic vein in 1979. One of three patients became transiently insulin independent nine months post-transplant (19, 20). However, this achievement lacked the support of C-peptide levels, was not well documented and was difficult to replicate.

Progress in the field of islet transplantation continued at a slow pace. The development of a semi-automated method for islet isolation by Camillo Ricordi and continuous density gradients for purifying the pancreatic digest to yield pure islets were major steps in advancing clinical islet transplantation (FIGURE 1-2) (21-24). Based on these improvements, the St. Louis group demonstrated in 1989 its first case of insulin independence following islet allograft into the portal vein in a patient with T1DM. But after only 22 days, blood glucose levels began to rise and insulin was reinstated after 25 days (25, 26). The Pittsburgh group quickly followed in 1990 with the first successful series of human islet allografts using a novel steroid-free, tacrolimus-based immunosuppressive regimen in patients without autoimmunity. Insulin independence at one year was achieved in over 50% of patients receiving combined liver and islet allografts following upper abdominal exenteration for malignancy (27).

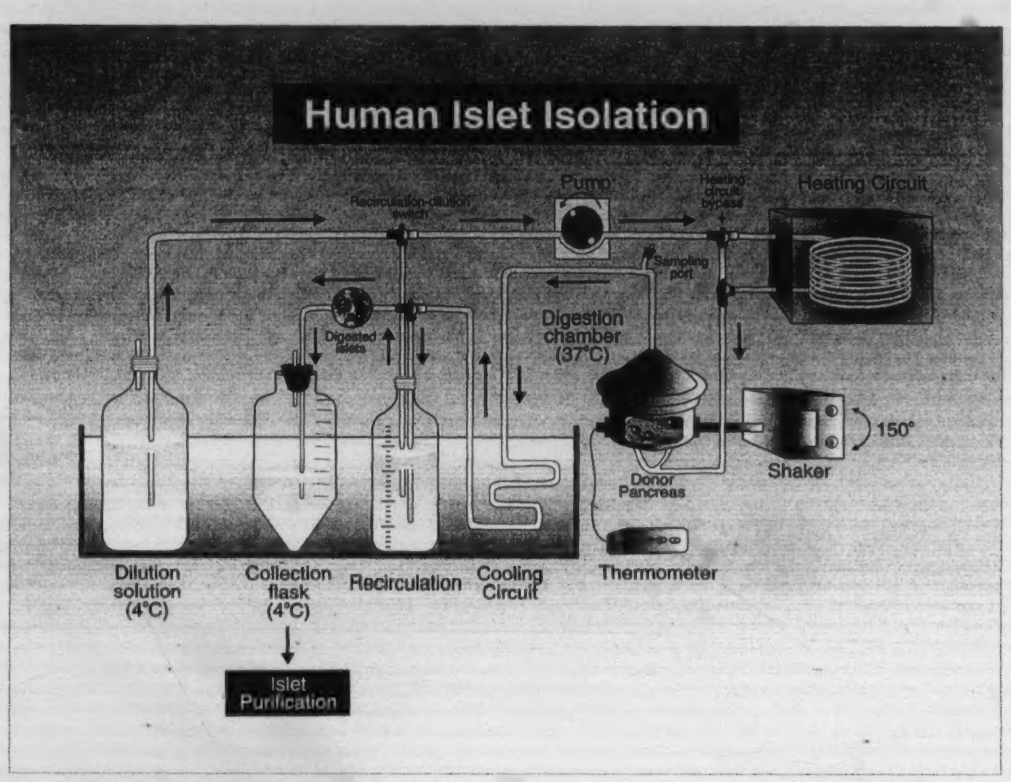

Human Islet Isolation
Recirculation-dilution switch
Pump
Heating circuit bypass
Heating Circuit
Sampling port
Digested islets
Digestion chamber (37°C)
150°
Shaker
Donor Pancreas
Thermometer
Dilution solution (4°C)
Collection flask (4°C)
Recirculation
Cooling Circuit
Islet Purification

Figure 1-2 Human Islet Isolation
The dissociation chamber consists of two compartments separated by a mesh. The lower cylindrical portion has two inlets on its base and receives the pancreatic tissue and marbles; the upper conical portion collects the pancreatic digest and has an outlet at its apex. Enzymatic digestion is carried out at 37°C and is enhanced by mechanical action of the marbles in the chamber that is shaken throughout the dissociation process. A tube system connected to the chamber and peristaltic pumps facilitate the circulation of the enzyme from the base of the chamber through the mesh towards its apex. The sampling port allows assessment of islet integrity to decide when to stop the enzymatic digestion.

Encouraged by these initial reports, the clinical islet programs in Edmonton, Giessen, Milan, Minnesota and St. Louis began intensive efforts to develop the first reproducible islet transplant protocol for T1DM. From 1977 to 1990, a series of patients received intraportal islet auto-transplantation after total or near-total pancreatectomy for chronic pancreatitis and nine of 22 demonstrated insulin independence for at least several months (28). Simultaneous and post-renal transplant patients with T1DM were treated with islet allografts in a series of transplants in 1991 (29). Although some patients were insulin independent for a brief time, all eventually required reinstatement of insulin. Insulin independence was reported in a small series in 1993 using the agent 15-deoxyspergualin, along with antilymphocyte globulin, for induction immunosuppression, and azathioprine, prednisone, and cyclosporine for maintenance in simultaneous single donor islet and kidney transplants (30). To determine what factors may indicate rejection in simultaneous islet-kidney transplants, islet cell antibodies and glutamic acid decarboxylase (GAD-65) antibodies were studied in seven patients in 1996 (31).

During the 1990s over 50% of the 267 islet allografts performed in patients with T1DM failed within two months with only 35% having continued graft function when measured by C-peptide secretion and 8% insulin independence one year after transplant. Despite these unimpressive results, functioning islet grafts provided superior glycemic control with effective prevention of recurrent hypoglycemia when compared to intensive insulin therapy (32). By the end of the decade the group in Giessen achieved continued graft function in up to 50% of patients, but less that 20% of recipients showed insulin independence at one year, using steroids, cyclosporine and azathioprine or mycophenolate-based immunosuppression (33).

Sustained insulin independence with islet transplantation remained elusive until July 2000 when the Edmonton group reported 100% insulin independence in seven consecutive patients with T1DM receiving islet transplants (34). The results rejuvenated the field of islet transplantation. Based on an in-depth analysis of the cumulative world experience in human islet transplantation (33), refinements in processing, optimization of the number of islets infused, and novel immunosuppression formed the foundation for the Edmonton Protocol. In 2001, the Immune Tolerance Network (ITN) with support from the Juvenile Diabetes Research Foundation (JDRF) began an international trial in nine centers to replicate the Edmonton protocol in 40 recipients. Results from the ITN study have confirmed that Edmonton's success is reproducible. However, when the ITN study is taken together with the results of other centers the insulin independence rates varied based on the center's previous experience both with islet isolation and in the clinical use of sirolimus-based immunosuppression. The one year insulin independence rates ranged from 23% (average of six newer centers with success rates of between 0 and 63%) to 82% (at the three most experienced centers) (FIGURE 1-3) (35, 36).

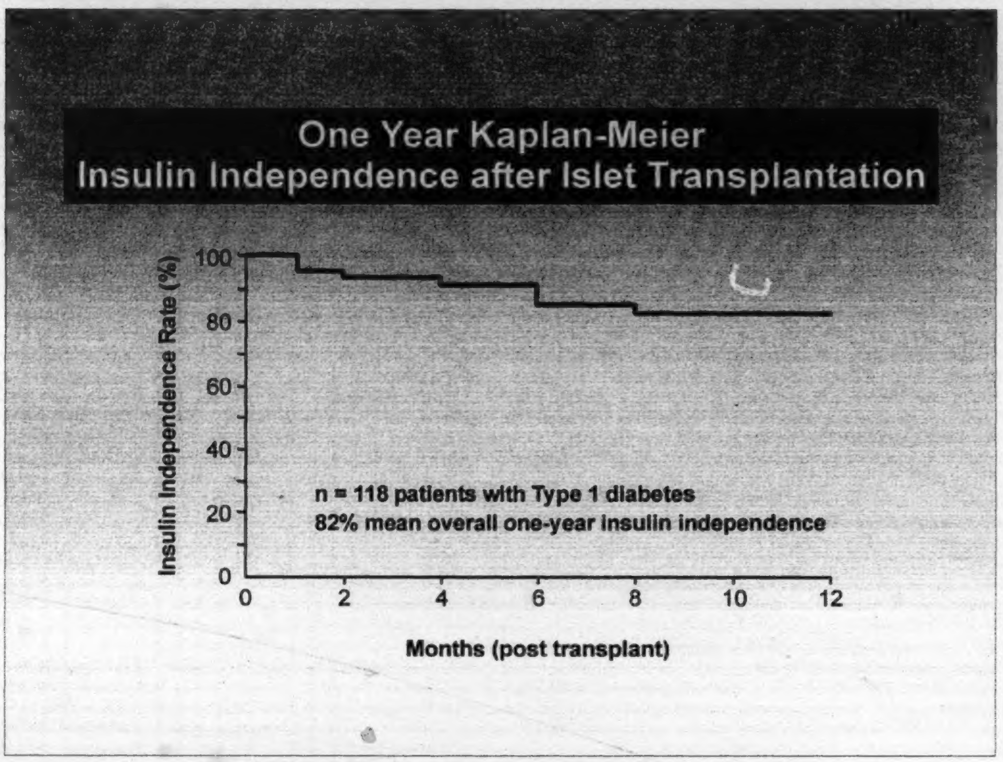


Figure 1-3 Insulin Independence One Year after Islet Transplantation
Insulin independence rates at one year after islet transplantation continue to be impressive at the three most active North American institutions. A recent combined analysis showing 82% of a total 118 recipients in Edmonton, Miami, and Minnesota were insulin free at one year.

1.4.1 Improvements in islet isolation & purification

Current islet isolation techniques evolved from incremental improvements accumulated through strong international collaborative efforts over the decades since the introduction of collagenase digestion of the pancreas in 1967.

The need for a standardized collagenase preparation was met with the development of Liberase™ (Roche Diagnostics), which is a purified blend of collagenase isoforms I and II from *Clostridia histolyticum* and non-clostridial protease that are low in endotoxin (37, 38). Intraductal collagenase delivery into the pancreas decreased direct exposure of the islets to digestive enzymes while increasing digestion of the exocrine pancreas. This technique greatly improved islet yield and purity (39) and was standardized with pressure-controlled perfusion (40).

The use of density gradients involving Ficoll® (a high molecular weight 400 kDa polymer of sucrose) facilitated the separation of islets from acinar tissue. In 1973, when Ficoll® was dialyzed before centrifugation to remove its low molecular weight, osmotically active contaminants, islet recovery improved (41). In 1991, Euro-Collins introduced a cold, hypertonic-density preservation solution as a vehicle for dissolving Ficoll® powder, which prevented edema of the exocrine tissue allowing better separation from islets (42). Use of a COBE 2991 cell separator (23) facilitated large-scale continuous density gradient centrifugation to separate islets from exocrine tissue (FIGURE 1-4).

The development of a semi-automated method of islet isolation in 1986 by Camillo Ricordi facilitated continuous monitoring of enzymatic digestion and collection of increased numbers of human islets (43). With the subsequent introduction of the zinc-

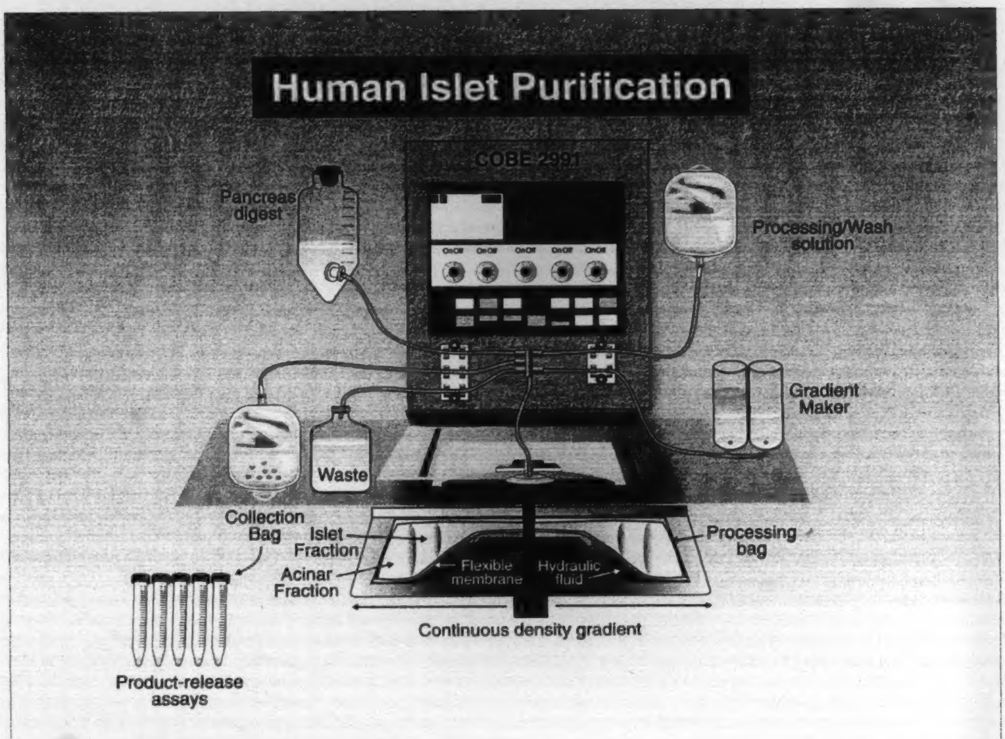

Human Islet Purification
COBE 2991
Pancreas digest
Processing/Wash solution
Gradient Maker
Waste
Collection Bag
Islet Fraction
Acinar Fraction
Flexible membrane
Hydraulic fluid
Processing bag
Continuous density gradient
Product-release assays

Figure 1-4 Human Islet Purification
The crude pancreatic slurry is then concentrated by centrifugation, and purified using continuous gradients of Ficoll-diatrizoic acid (Seromed-Biochrom) on a refrigerated Cobe 2991 centrifuge (Cobe BCT, Lakewood, CO) to separate the islets from other products of digestion including exocrine tissue. The product is centrifuged at 2400 rpm for 5 minutes, while the temperature of the preparation is maintained at 4 to 10°C during this process either by core cooling of the Cobe 2991 or by carrying out the purification in a specially designed cold room (23).

chelating agent dithizone, which stains islets bright red, further enhanced islet visualization and purification (44).

Using these techniques significantly improved overall islet quality and it became possible to reliably isolate enough viable islets for clinical transplantation. When these processing changes were combined with improvements in immune suppression, early graft survival significantly improved (45, 46).

1.4.2 Advances in immunosuppression

The evolution of immunosuppression in the last five years has seen a decline in the use of corticosteroids. Sirolimus-based regimens allowed steroids to be rapidly tapered and completely withdrawn by 3 months in solid organ transplantation. Although effective for immunosuppression, corticosteroid exposure is associated with insulin resistance and islet exhaustion. McAlister and colleagues from Halifax, Nova Scotia, Canada developed an immunosuppression regimen based on sirolimus, low-dose tacrolimus, steroids and antithymocyte globulin induction (47). As a result, recipients of pancreas grafts and various other solid organ grafts demonstrated low rates of rejection and excellent graft function (47). The proposal to eliminate corticosteroids from the anti-rejection drug mix and to cluster liver-islet transplants was initiated in the early to mid-1900s by a number of investigators, including Dr. Camillo Ricordi at the University of Pittsburgh, PA, USA and also the Milan Group, in order to reduce the diabetogenic effect of the immunosuppression (27). The effectiveness of steroid-free immunosuppression was demonstrated in a study with islet-kidney transplantation (48). The Edmonton group substituted an inductive course of an antibody to the IL-2 receptor (anti-CD25, daclizumab) for antithymocyte globulin and steroids. Daclizumab, in addition to effective blockade of IL-2 with sirolimus, inhibits T-cell activation and provides potent immunosuppression.

While the combined use of sirolimus and low-dose tacrolimus has helped move islet transplantation forward from clinical curiosity to more effective therapy for many patients, these drugs are far from ideal. Sirolimus is associated with lipid abnormalities but not usually glucose intolerance, and may in fact enhance insulin secretion (49-51), however tacrolimus has been shown to have direct β cell toxicity (52-54). Both sirolimus and tacrolimus have near-ubiquitous targets of distribution, which leads to a number of side effects in islet recipients, including: mouth ulceration, peripheral edema, a high rate of ovarian cysts in female recipients, hypertension, hypercholesterolemia, and increase in proteinuria in some patients with underlying pre-existing diabetic renal damage (55, 56). Lifelong immunosuppression remains a major limitation to the broad application of islet transplantation, and careful consideration of the cost-benefit analysis for each patient is required.

1.4.3 Islet culture & handling

The initial Edmonton report used islets immediately after isolation rather than frozen or cultured islets as had been historically done (34). At that time, prolonged culture of islets was associated with loss of endocrine cells (57). However, improvements in islet isolation and handling allowed for islets to be cultured successfully for several days prior to transplantation without the loss of viability (58). Developments included: (i) improved transport media (59); (ii) the use of the two-layer perfluorochemical (PFC) organ preservation method (FIGURE 1-5) (60, 61); and (iii) emerging use of an alternative gradient media (iodixanol; Opti-Prep^{TM}, Axis-Shield, Oslo, Norway), although it is still not clear whether these non-ionic gradient media are less toxic than Ficoll®-based media (58).

These advances in islet culture and pancreas preservation have made islet transplantation more available and geographically accessible. The concept of central islet processing facilities was established in the mid- 90s (62). Cultured islets allowed the development of centralized islet processing centers, which has been successfully demonstrated by the Baylor and Miami groups who have flown harvested pancreases from Houston to Miami for islet isolation and back to Houston for transplantation (59). In Europe, similar collaborative networks with central islet processing facilities have been

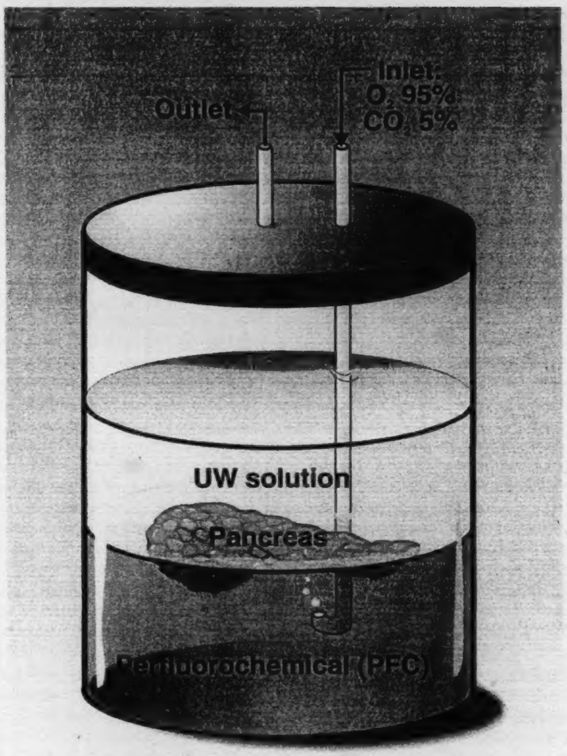


Figure 1-5 The Perfluorochemical (PFC) 'two-layer' Method for Pancreas Preservation

developed in Geneva for the Swiss-French GRACIL (Groupe Rhin-Rhone-Alpes-Geneve pour la Transplantation d'Ilots de Langerhans) collaborative network and at the Uppsala center in Sweden for central islet processing for the Nordic network of islet transplant centers.

Cultured islets also improve safety, since transplants can be scheduled electively, and time is afforded for pretransplant conditioning or other immunosuppressive therapy. Although there is some loss of islets during the culture period, there is an increase in islet purity due to a more rapid loss of exocrine tissue. As a result, along with associated reduction in volume of tissue infused there is a theoretical reduction in risk of portal hypertension and portal vein thrombosis.

1.4.4 Donor selection

Islet isolation success depends on donor selection and careful surgical technique during organ procurement. Donor criteria for islet isolation are similar to those for solid organ pancreas transplantation but a number of variables appear to be unique to islets (TABLE 1-1) (63). Pancreases from older donors with a high body mass index (BMI) appear to have a higher rate of successful isolation, and these donors are less suitable for whole pancreas transplantation. While there is potential for competition for good pancreas donors between whole pancreas and islet transplant programs, it is possible for these two activities to co-exist successfully within the same institution.

Table 1-1 Donor-related variables predicting isolation success (Lakey et al.(63)).

Variable	P-value	R-value	Odds Ratio*
Donor age (years)	**<0.05**	**0.18**	**1.10**
Body mass index	**<0.01**	**0.19**	**1.30**
Local vs. distant procurement team	**<0.01**	**0.21**	**7.04**
Min. blood glucose	**<0.01**	**-0.24**	**0.68**
Duration of cardiac arrest	**<0.01**	**-0.17**	**0.81**
Duration of cold storage	**<0.05**	**-0.13**	**0.86**

*Odds ratio >1, positive correlation with isolation success; < 1, negative correlation.

1.4.5 Islet assessment

The resulting purified islet preparation is placed in culture, washed, counted and assayed to determine whether it meets defined product release criteria before releasing to a potential recipient. Product release criteria are essential to maximize safety, and to ensure that a pure, viable and functional islet preparation is consistently being produced for transplantation. These criteria include: islet yield, sterility, endotoxin load, purity, and viability (TABLE 1-2).

Table 1-2 Islet Product Release Criteria

Assessment	Method	Required Result
Islet yield	**Count of IEQ on aliquots of the final preparation**	**≥ 5000 IE/kg (weight based on recipient)**
ABO blood group and Cross Match	**ABO type and Cross Match**	**ABO-compatibility and negative donor-recipient cross match**
Sterility	**Stat Gram stain**	**Negative**
Endotoxin load	**Endotoxin concentration**	**< 5 endotoxin units (EU/kg)**
Islet volume	**Packed cell volume**	**< 5 cc (normally 2-3 cc)**
Purity	**Dithizone staining**	**> 30% (usually > 50%)**
Viability	**Fluorescein diacetate/propidium iodide SytoGreen/ethidium bromide**	**> 90% (FDA/PI) > 70% (SytoEb)**

Dithizone staining is used to identify islets for their enumeration in estimating islet yield, integrity, and purity after isolation and culture (44). Dithizone binds zinc in secretory granules staining the endocrine cells a characteristic red color (44). Islet numbers are quantified and scored in duplicate by size using an algorithm to calculate islet equivalents (IEQ). An IEQ is defined as the ideal spherical islet with a diameter of 150 µm (64, 65). The process of islet counting is not an accurate process, as sampling errors in taking the 200 µL aliquots may be further compounded by errors in the counting process which is usually done manually under the microscope. Furthermore, islets greater than 350 µm in the sample have substantial impact on the final calculated islet yield, and islets exceeding this diameter may fail to engraft optimally or may develop central necrosis after transplantation due to limited oxygen diffusion capacity.

Safety is assessed by Gram stain and endotoxin assay. Sterile conditions are determined by a negative Gram stain and endotoxin levels in the media used for islet isolation and culture should be < 5 endotoxin units (EU)/kg, based on the recipient's body weight. Assessment of islet viability is generally performed visually by the use of membrane dye exclusion methods, such as fluorescein diacetate/ propidium iodide (FDA/PI) or SytoGreen/ ethidium bromide (SytoEb), that allow for the discrimination of viable and dead cells (66, 67). The FDA/PI stain is insensitive, and it is very rare to observe values less than 95%. The SytoEb stain is much more sensitive however, and more accurately reflects the likelihood of an islet preparation to remain intact during subsequent culture (67). Islet viability must exceed 70%. Islet function is routinely measured using glucose-stimulated insulin release assays (65, 68-70). A Stimulation Index (SI) is calculated, based on insulin release into the surrounding media during high (20 mM) compared with low (2.8 mM) glucose exposure. While this assay confirms that an islet preparation can release insulin, unfortunately the magnitude of the response fails to correlate with clinical outcome. Currently an SI >1.0 is used to confirm that the islet product is suitable, but this test is usually only available *post hoc*, after the islets have been transplanted. When a period of islet culture is used, it is possible to run the SI test on the day of transplant. Newer and more sensitive dynamic assays of islet functional viability are needed, that will correlate more accurately with clinical potency. Islet oxygen consumption rate and β cell ATP content assays are in development that may more accurately reflect *in vivo* islet function, but are time consuming and expensive. A alternative approach is to transplant small aliquots of the final islet preparation into diabetic immunodeficient mice (58, 71-73). The drawback of this approach is again that the assay read-out (reversal of diabetes in the mouse) takes some days to determine, and again therefore is only available *post hoc* (68, 71-74). This approach in combination with glucose tolerance tests in transplanted animals can be used to validate new *in vitro* tests for predicting human islet quality (74-78). The Miami group have recently developed a colorimetric assay using laser-scanning microscopy and TMRE dyes, and this approach looks promising as a rapid, *in vitro* predictive assay (78).

1.4.6 Patient selection for islet transplantation

Islet autotransplantation has been performed for almost three decades in patients who required total or near-total pancreatectomy for chronic pancreatitis (28, 79). T1DM patients are selected for islet transplantation either as an islet-transplant alone (ITA), or as an islet-after solid organ transplant (usually kidney, IAK). The decision to transplant a patient that is already taking chronic immunosuppression to protect a kidney graft is usually straightforward, as the patient already faces the chronic risks attendant to immunosuppression. The decision to initiate life-long immunosuppression for an ITA patient is much more complex however, and requires careful justification. Current major indications for ITA include hypoglycemic unawareness and metabolic lability, where repeated attempts have been tried and failed to stabilize control through optimization of insulin regimens (TABLE 1-3).

More objective scoring systems have been created to quantify the severity of hypoglycemia and glycemic lability, and these tools have been used to screen potential candidates for islet replacement therapy. In the past the mean amplitude of glycemic excursion (MAGE) used 14 blood glucose values over a 2 day period to assess potential recipients (80, 81); however, it has now been superseded recently by the HYPO score and lability index (LI), developed by Ryan et al., and is based on 4 weeks of glucose values (82, 83). The HYPO score considers the frequency, severity and degree of hypoglycemia unawareness. Problematic hypoglycemia is associated with a HYPO score ≥ 1047 (82). Labile diabetes is defined as frequent, wide variations in blood glucose that interfere with patient lifestyle and is characterized by a mean amplitude of glycemic excursion > 11.1 mM (81) and a lability index (LI) of ≥ 433 mM^2/h/week (82). Generally a patient must have either a HYPO score or LI above the 90th percentile based on the general type 1 diabetic population (≥ 1047 and ≥ 433 mM^2/h/week, respectively) or have both scores above the 75th percentiles (423 and 329 mM^2/h/week, respectively). These scores provide objective methods of assessment that allow comparison pre and post islet transplantation. Contraindications to islet transplantation include, but are not limited to: unstable coronary artery disease, the presence of active proliferative retinopathy, or macroproteinuria ≥ 1 g/day.

Table 1-3 Selected Patient Eligibility and Exclusion for Islet-alone Transplantation

Indications	Exclusion
• **T1DM** • **Negative Stimulated C-peptide (<0.3ng/ml)** • **Intensive diabetes management**[a] • **One or more severe hypoglycemic events**[b] • **Plus one of the following three:** ▪ **Reduced awareness of hypoglycemia** [c] ▪ **Marked glycemic lability**[d] ▪ **Composite score > 75th percentile**[e]	• **HbA1c > 10%** • **Untreated proliferative retinopathy** • **BP > 160/100** • **GFR < 70 (females) or < 80 (males) ml/min/1.73 m^2** • **Present or history of macroalbuminurea** • **Panel reactive antibodies (PRA) > 20%** • **Severe cardiac disease** • **Desired pregnancy (female recipients)** • **Patient satisfaction with current management**

[a] Glucose testing 3 or more time/day, 3 or more insulin injections per day or on insulin pump, directed by an endocrinologist, diabetologist, or diabetes specialist.
[b] In the past 3 years requiring assistance, blood glucose < 50 mg/dl, IV glucose or glucagon
[c] HYPO score ≥ 90th percentile (≥1047) within recent 6 months
[d] Wide swings in blood glucose levels despite optimal diabetes therapy, defined as a glycemic lability index ≥90th percentile (≥ 433 mM^2/hr/week) within or recent 6 months.
[e] HYPO score ≥ 75th percentile (≥423), and LI ≥ 75th percentile (≥329 mM^2/hr/week)

1.4.7 Transplantation procedure

After obtaining informed consent, the patient is admitted to hospital for the following laboratory investigations: complete blood count (CBC), chest x-ray, liver function tests (LFTs), and coagulation screen. Initially patients transplanted under the

Edmonton Protocol received islet infusions immediately after purification rather than frozen or cultured islets as had been done previously (6). At that time, prolonged culture of islets was associated with loss of endocrine cells (57). However, improvements in islet isolation and refinements in the constituents of the media solutions allowed for islets to be cultured successfully for several days prior to transplantation without the loss of viability (58, 66, 84). Cultured islets may improve both the safety and practicality of islet transplantation, as: 1) transplants can be scheduled electively; 2) product release testing can be more complete; 3) time is afforded for pre-transplant recipient immunological conditioning; 4) although there may be a mean of up to 20% islet loss during culture, islet purity generally increases and the packed tissue volume falls due to preferential loss of exocrine tissue, which may reduce the risk of portal vein thrombosis. The majority of our islet preparations (97/140, 69%) in Edmonton have been placed in culture (22°C and 5% CO_2) for a median of 13.0h before transplantation to facilitate timing of islet infusion or as part of the immunosuppressive protocol (85).

The islet preparation may be infused into the portal vein using either a surgical or radiological approach. The percutaneous approach cannot be used if hepatic hemangiomas are present or if anticoagulants cannot be discontinued before transplant. In the surgical approach, a mini-laparotomy is performed under general anesthesia, and a catheter is placed in a tributary of the portal vein. Our choice, if the surgical approach is required, is to place a 9Fr dual lumen Broviac catheter into a branch of the middle colic or inferior mesenteric vein. This approach facilitates access to the portal vein while allowing simultaneous continuous monitoring of the portal venous pressure through the second catheter lumen. The less invasive, radiological approach is however preferred for most of our patients as it avoids a need for surgery, incisional discomfort and general anesthesia. In the Department of Radiology, percutaneous portal vein cannulation is performed under a combination of ultrasound and fluoroscopic guidance (86, 87). A 7Fr catheter is threaded usually from a right-sided approach down in to the main portal vein, and its position confirmed by fluoroscopy. The baseline portal pressure is then monitored, and if <18 mmHg, the islet preparation is gently infused in approximately 250 ml suspension of transplant media after addition of heparin to the transplant bag (88). It is now generally recommended that 70 units per kg heparin be mixed in the islet bag prior to islet infusion, and if there are no concerns relating to the radiological approach and tract management, systemic heparinization should be continued in the post-transplant period (500 units per hour) for up to 48 hours, to minimize the risk of portal vein thrombosis. Prior to 2001, a syringe was used to infuse islets; however, the 'bag technique' allows the maintenance of islets in sterile suspension and lowers the risk of elevated portal pressure, which is monitored at multiple times during and after infusion (88). We previously encountered bleeding from the liver surface catheter tract on occasion after withdrawal of the infusion catheter, necessitating either blood transfusion or surgical intervention (89, 90). This complication is now regarded as completely preventable, if methods are used to plug the entire length of the catheter egress track. Effective approaches have included the placement of thrombogenic viscous solutions in the track (e.g. Avitene paste, tissue fibrin glue (Tisseel) or D-Stat), or previously by use of multiple coils and gelfoam micro-pledgets. Our current preference is to use Avitene paste, made up using 1g of Avitene powder mixed with 3cc of Omnipaque contrast and 3cc of saline. This creates a thick paste that is readily visible on fluoroscopy, and safe placement without risk of central embolization can therefore be achieved. Using this approach, we have not encountered bleeding complications in our most recent experience.

Hourly blood glucose levels are monitored initially post infusion and insulin therapy is instituted only when fasting capillary glucose increases to > 6.0 mM or > 8.0 mM at two hours postprandial. Patients are usually discharged the following day once the CBC and LFTs are acceptable, and an ultrasound has excluded portal vein thrombosis or intraperitoneal bleeding. Aspirin (81 mg/day for 14 days) and enoxaparin (30 mg b.i.d. s.c. for 10 days) are given once major bleeding has been excluded.

Repeated islet transplants may be offered at a subsequent time point to augment the islet engraftment mass, and a patient is deemed to be 'complete' either when insulin independence is achieved, or after two (or very rarely three) islet infusions. Insulin independence is defined as no use of exogenous insulin for 4 weeks and no more than two capillary glucose levels > 10.0 mM per week, and a HbA_{1C} < 6.5%.

1.4.8 Microbial Prophylaxis

Sulfamethoxazole/ trimethoprim (SMX/TMP) is used for *Pneumocystis carinii* prophylaxis for 6 months. Cytomegalovirus (CMV) prophylaxis with gancyclovir 1 000 mg t.i.d is used for 3 months in patients who are CMV negative and receiving islets from CMV positive donors.

1.4.9 Follow-up & evaluation of graft function

Drug levels and routine laboratory blood work including: CBC, LFTs, electrolytes, calcium, and magnesium, are measured three times a week for the first 2 weeks, twice a week for the next 2 weeks, weekly for the next month, and then every 2 weeks for a month.

For long-term post transplant follow-up, patients are seen monthly or at least every 6 months depending on geographical location and access to the transplant centre. During each visit, glucose control records and adverse events are reviewed along with weight and blood pressure determination. The lipid profile, LFTs, electrolytes, calcium, magnesium, and CBC are measured together with fasting glucose, insulin, and HbA_{1C}. Islet cell and insulin antibodies are also determined. Every 6 months, a full physical examination and a fasting meal tolerance test are performed. The physical exam includes an assessment of neuropathy with a neurothesiometer. For the fasting meal tolerance test, patients are given oral Ensure High Protein at 6ml/kg to a maximum of 360 ml, providing 391 kcal with 8.5 g fat, 44 g carbohydrate, and 17 g protein and blood is drawn for baseline and 90 min glucose and C-peptide levels. The HYPO score and LI are determined yearly (82), as is a composite measure of graft function, the β score (83). In Edmonton a composite β score was developed as a simple index of global islet function in islet transplant recipients, based on the fasting blood glucose, HbA1c, insulin requirement and stimulated C-peptide response (TABLE 1-4) (83). Homeostasis model assessment (HOMA) is calculated for an estimation of insulin sensitivity (91, 92).

Table 1-4 Determination of Components of the β Score (Ryan et al., (83)).

Components	Score of 2	Score of 1	Score of 0
Fasting Plasma Glucose (mmol/l)	< 5.6	5.6-6.9	≥ 7.0
HbA1c	< 6.2	6.2-6.9	≥ 7.0
Daily insulin (units/kg) or OHA use	-	0.01–0.24 and/or OHA use	≥ 0.25
Stimulated C-peptide (nmol/l)	≥0.3*	0.1-0.29	<0.1†

*If fasting C-peptide was 0.3 nmol/l, then the stimulated C-peptide level was assumed to be 0.3 nmol/l.
†If stimulated C-peptide was <0.1 nmol/l, then an overall score of 0 was awarded.

Insulin is restarted for fasting capillary glucose > 8.0 mM, or glucose > 10.0 mM at two hours postprandial, and/or the HbA1c is sustained > 6.5%. Loss of islet graft function is defined as two stimulation tests showing C-peptide levels below the level of detectability of the assay (0.1 nM), or if the fasting glucose is > 15.0 mM with no measurable C-peptide. Plasma glucose concentrations are determined by the glucose oxidase method. Panel reactive antibodies (PRAs) as a measure of HLA-related sensitization are detected with anti-human globulin and by flow cytometry.

1.5 CURRENT STATUS OF CLINICAL ISLET TRANSPLANTATION

More patients with T1DM have now received islet transplants in the past 5 years than in the entire preceding 30-year history of islet transplantation. Since 2000, there has been an unprecedented worldwide exponential increase in islet transplantation activity. It is now estimated that over 500 islet transplants have been performed at more than 50 international centers (FIGURE 1-6) (93).

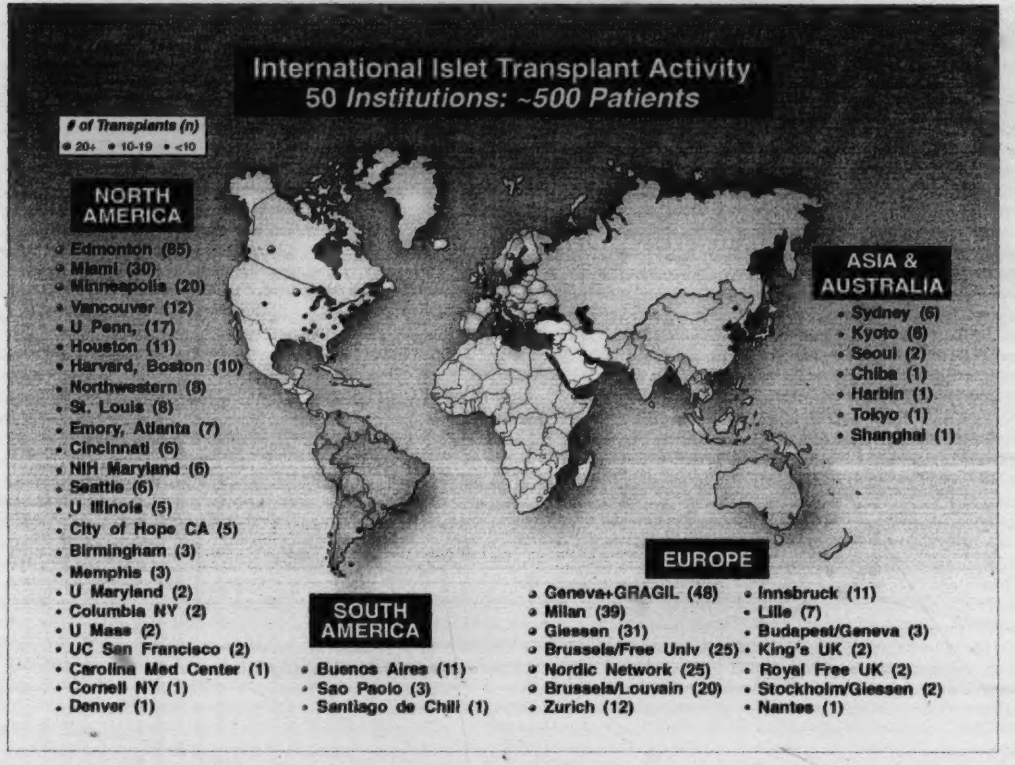

International Islet Transplant Activity
50 Institutions: ~500 Patients
# of Transplants (n)
● 20+ ● 10-19 • <10
NORTH AMERICA
Edmonton (85)
Miami (30)
Minneapolis (20)
Vancouver (12)
U Penn, (17)
Houston (11)
Harvard, Boston (10)
Northwestern (8)
St. Louis (8)
Emory, Atlanta (7)
Cincinnati (6)
NIH Maryland (6)
Seattle (6)
U Illinois (5)
City of Hope CA (5)
Birmingham (3)
Memphis (3)
U Maryland (2)
Columbia NY (2)
U Mass (2)
UC San Francisco (2)
Carolina Med Center (1)
Cornell NY (1)
Denver (1)
SOUTH AMERICA
Buenos Aires (11)
Sao Paolo (3)
Santiago de Chili (1)
EUROPE
Geneva+GRAGIL (48)
Milan (39)
Giessen (31)
Brussels/Free Univ (25)
Nordic Network (25)
Brussels/Louvain (20)
Zurich (12)
Innsbruck (11)
Lille (7)
Budapest/Geneva (3)
King's UK (2)
Royal Free UK (2)
Stockholm/Giessen (2)
Nantes (1)
ASIA & AUSTRALIA
Sydney (6)
Kyoto (6)
Seoul (2)
Chiba (1)
Harbin (1)
Tokyo (1)
Shanghai (1)

Figure 1-6 International Islet Transplant Activity
An unprecedented worldwide exponential increase in islet transplantation activity has occurred over the last five years since the year 2000, with more patients, with type 1 diabetes, receiving islet transplants than in the previous 30-year history.

As of August 2005, the Edmonton group has performed over 140 islet transplants in over 85 patients under the Edmonton protocol and more recent collaborative refinements, with initial insulin independence expected in 70 to 80% of recipients at one year after delivery of between two to four islet infusions. Under sirolimus and tacrolimus-based maintenance therapy, Kaplan-Meier statistical projections demonstrate that less than 50% of recipients remain insulin free at three years after islet transplant, and only 11% of recipients at five years (85). While insulin independence rates inexorably decline in most patients over time, 82% have continued islet graft function at 5 years when detected by C-peptide levels (FIGURE 1-7).

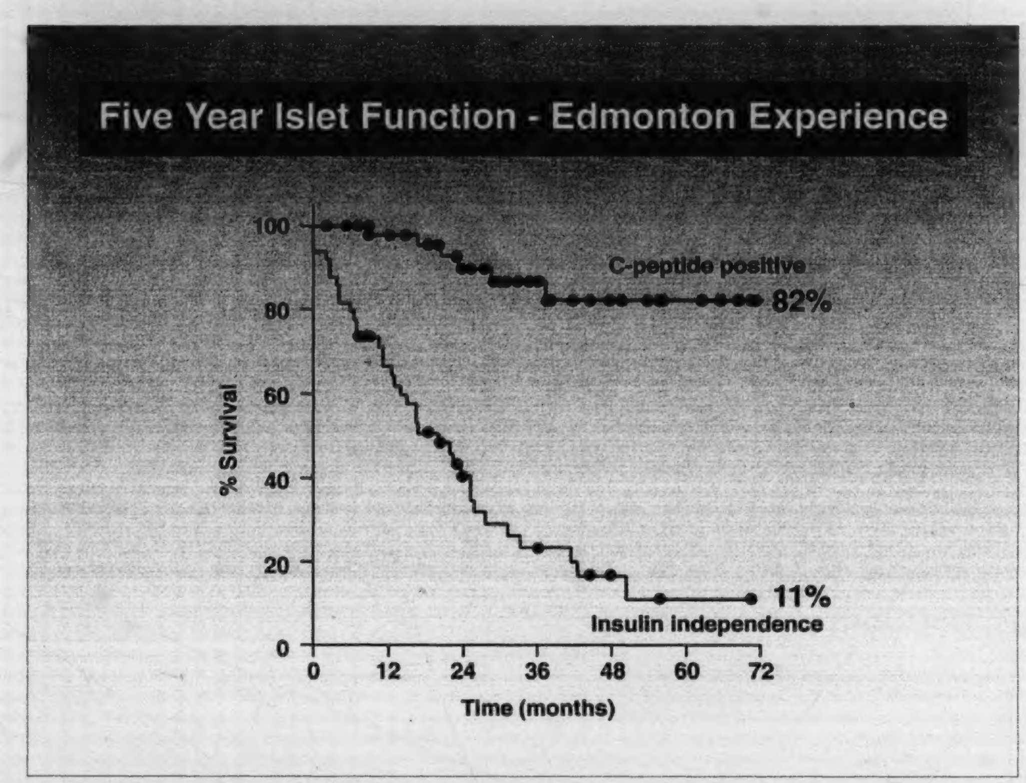


Figure 1-7 Islet Function at Five Years at the University of Alberta, Edmonton
Inexorable decline in insulin independence rates occur over time and Kaplan-Meier statistical projections show that less than 50% of recipients remain insulin free at three years after islet transplant, and only 11% of recipients at five years (85). While C-peptide secretion rates are as high as 82% at 5 years (reproduced from Ryan et al. (85), with permission from The American Diabetes Association, Copyright © 2005 American Diabetes Association).

Patients with persistent islet graft functions in the absence of insulin independence continue to benefit from improved HbA_{1c} values, protection from recurrent hypoglycemia, and sustained correction of glycemic lability far superior to that readily achievable with intensive insulin therapy (FIGURE 1-8) (85, 94, 95).

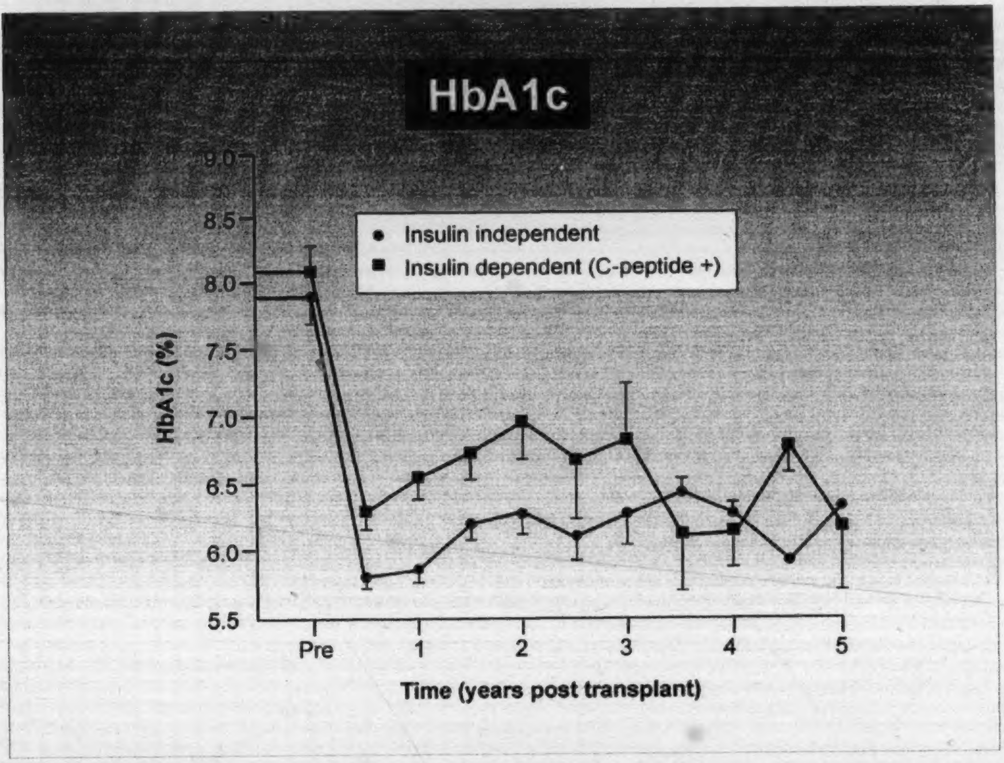


Figure 1-8 Glycosylated Hemoglobin (HbA_{1c}) Levels after Islet Transplantation
Insulin dependent patients with persistent islet graft function continue to benefit from improved HbA_{1c} levels far superior to that readily achievable with intensive insulin therapy (85, 94, 95), and comparable to insulin independent patients after islet transplantation (reproduced from Ryan et al. (85), with permission from The American Diabetes Association, Copyright © 2005 American Diabetes Association).

The Milan group have clearly shown that persistent C-peptide secretion after islet transplantation can improve cardiac, renal, and neurologic function, and can improve patient survival in islet-kidney transplant recipients (96-98). In Edmonton a composite "β score" was developed, as a simple index of global islet function in islet transplant recipients, based on the fasting blood glucose, HbA_{1c}, insulin requirement and stimulated

C-peptide response (83). Upon analysis of outcome data in terms of these parameters, graft function remains relatively stable over time and provides additional evidence for the benefit of islet transplantation despite the loss of complete insulin independence. The HYPO score and LI show marked improvement post-transplant (FIGURE 1-9). The patients who eventually require exogenous insulin experience episodes of hypoglycemia and some lability, but their HYPO and LI scores remain significantly improved for up to four years compared with values pre-transplant.

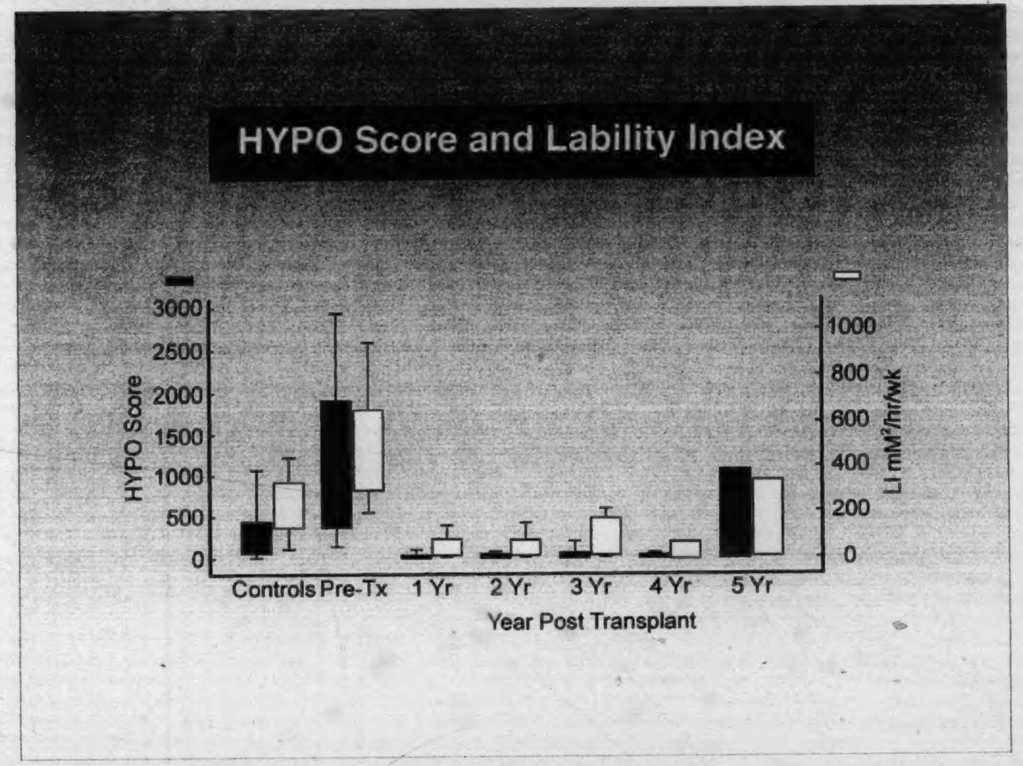


Figure 1-9 Function of Human Islet Allograft: HYPO Score and LI
The HYPO score and LI show marked improvement post-transplant. With the use of insulin there have been some episodes of hypoglycemia and more lability, but the scores remain significantly improved for up to 4 years compared with values pre-transplant (reproduced from Ryan et al. (85), with permission from The American Diabetes Association, Copyright © 2005 American Diabetes Association).

However, these benefits must be weighed against the actual and potential risks of current lifelong immunosuppression (TABLE 1-5) (55, 99, 100).

Table 1-5 Summary of Complications of Islet Transplantation under the *Edmonton Protocol* (85).

Procedural Complications	140 procedures in 65 patients
Bleeds	• 15 before Avitene
Partial thrombus within a portal branch	• 5 patients
Transaminitis	• >2x normal (55%) and > 5x normal (23%)
Gall Bladder perforation	• 2 punctures
Portal Hypertension	• 0
Fatty Liver	• 8 of 36 with MRI
Immunosuppression Complications	**Side Effect Rates**
Mouth Ulcers	• 89%
Diarrhea	• 60%
Acne	• 52%
Edema	• 43% (12% required change from sirolimus to mycophenolate mofetil)
Ovarian cysts	• Common in premenopausal women
Anemia	• 8% required Erythropoietin
Weight loss	• ~5 kg loss at 1 year
Pneumonia	• 3 patients, of which 1 was fungal infection
CMV infection	• 43 CMV-negative received CMV-positive islets (2 seroconverted)
Immunosuppression intolerance	• 33 (77%) remained on sirolimus and tacrolimus • 5 changed to tacrolimus and mycophenolate mofetil • 3 changed to sirolimus and mycophenolate mofetil • 2 changed to low-dose sirolimus and mycophenolate mofetil
Diabetes Complications	**Progression of Disease**
Retinopathy	• 4 of 47 progressed within 5 months
Nephropathy	• 5 of 11 progressed from microalbuminurea to macroproteinuria • 3 of 30 with no microalbuminurea progressed to macroproteinuria
Hypertension	• No antihypertensive: 36% pre-transplant to 5% post-transplant • > 1 antihypertensive: 6% pre-transplant to 42% post-transplant
Hyperlipidemia	• 23% to 83% on lipid lowering drugs
Neuropathy	• No significant increase as detected by neurothesiometer

1.6 CHALLENGES AND EMERGING OPPORTUNITIES

The decline in insulin independence rates observed in long-term follow-up using the *Edmonton Protocol* is a major challenge, but also a unique opportunity to better define and understand the biology involved in promoting and sustaining islet survival (FIGURE 1-10).

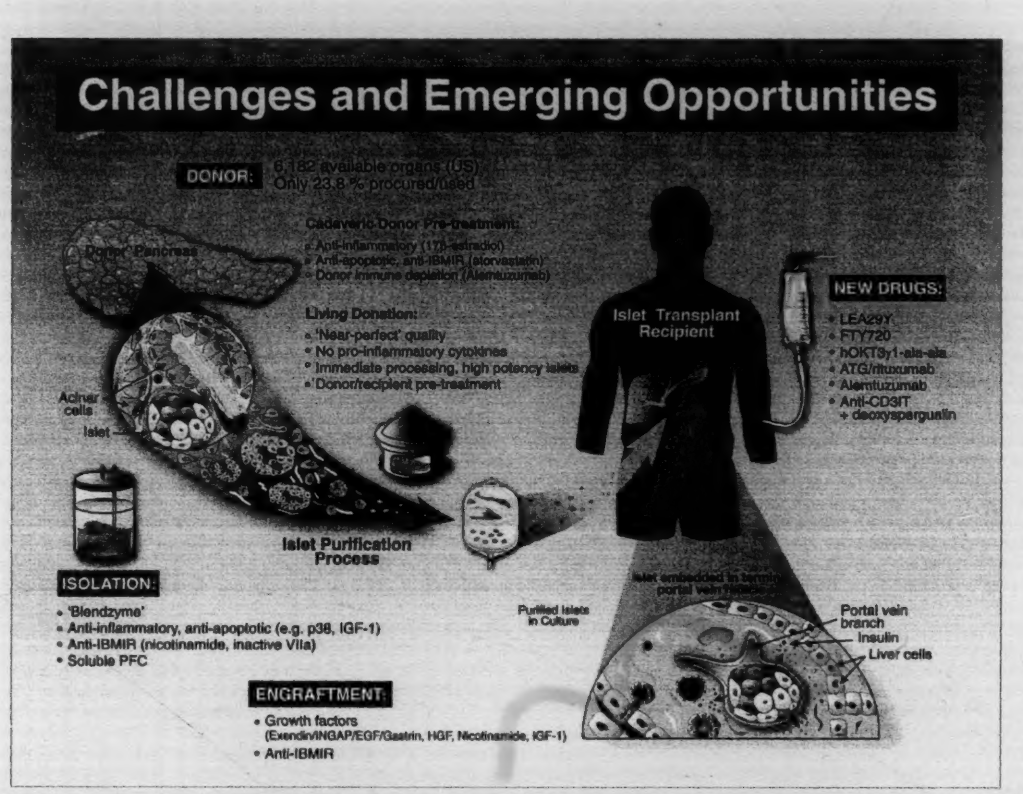

Challenges and Emerging Opportunities
DONOR:
6,182 available organs (US)
Only 23.8 % procured/used
Cadaveric Donor Pre-treatment:
• Anti-inflammatory (17β-estradiol)
• Anti-apoptotic, anti-IBMIR (atorvastatin)
• Donor immune depletion (Alemtuzumab)
Living Donation:
• 'Near-perfect' quality
• No pro-inflammatory cytokines
• Immediate processing, high potency islets
• Donor/recipient pre-treatment
Donor Pancreas
Acinar cells
Islet
Islet Purification Process
ISOLATION:
• 'Blendzyme'
• Anti-inflammatory, anti-apoptotic (e.g. p38, IGF-1)
• Anti-IBMIR (nicotinamide, inactive VIIa)
• Soluble PFC
ENGRAFTMENT:
• Growth factors (Exendin/INGAP/EGF/Gastrin, HGF, Nicotinamide, IGF-1)
• Anti-IBMIR
Islet Transplant Recipient
NEW DRUGS:
• LEA29Y
• FTY720
• hOKT3γ1-ala-ala
• ATG/rituxumab
• Alemtuzumab
• Anti-CD3IT + deoxyspergualin
Purified Islets in Culture
Islet embedded in termin
portal vein
Portal vein branch
Insulin
Liver cells

Figure 1-10 Challenges and Emerging Opportunities in Islet Transplantation
Many challenges remain before islet transplantation may be expanded to the general population with T1DM. However, these challenges may be seen as emerging opportunities to develop sustainable sources of islets, improve isolation and engraftment, enhance islet proliferation *in vitro* and in the recipient, and test newer immunomodulatory drugs with fewer side effects.

Results from an in-depth study of factors likely influencing islet mass decay using serial islet graft biopsies and serological analysis of donor sensitization, cytokine gene activity (granzyme B), and changes in autoantibody status will collectively provide valuable information. Possibilities for islet mass deterioration include chronic allograft rejection, undiagnosed acute rejection, recurrent autoimmunity, local islet toxicity from immunosuppressive drugs, or failure of islet regeneration secondary to the anti-proliferative properties of sirolimus (101).

1.6.1 Critical islet mass

The number of islets required for insulin independence was not initially known when the group in St. Louis attempted islet transplants in patients with T1DM in 1989. None of the recipients achieved insulin independence when islets from one donor (average of 6 161 islet equivalents (IEQ)/kg body weight) were infused. However, when islets from two or more donors were transplanted (average 13 916 IEQ/kg), two out of three recipients became insulin independent. Different approaches were explored over the next decade by investigators including the group in Edmonton, who believed that the islets from more than a single pancreas are needed to achieve insulin independence in patients with T1DM. On average 12,000 islet equivalents (IEQ)/kg were needed to achieve insulin independence in recipients.

1.6.2 Immediate post-transplant islet loss

Under current protocols, adequate islet mass requires islets from usually two, but up to four, separate donor infusions before insulin independence is obtained. However, approximately 10% achieved insulin independence with a single islet infusion. Transplanted islets correspond to only about 20% to 40% of the functional capacity in a normal pancreas (94, 102). Most investigators agree that between 50% and 70% of the islets will be lost immediately after experimental transplantation and only a small fraction successfully engraft (103, 104). A series of reports demonstrate that an instant blood-mediated inflammatory reaction (IBMIR) occurs within minutes when islets come in direct contact with ABO compatible blood (105-107). Platelet binding to and activation on the islet surface, in addition to the activation of the coagulation and complement systems characterize this phenomenon. Inflammatory mediators (such as tissue factor (TF) and MCP-1) expressed by isolated human islets induce leukocyte infiltration with subsequent disruption of islet integrity and islet loss (106, 108). Korsgren and colleagues from Uppsala have found that IBMIR may be a leading cause for the failure to achieve insulin independence after portal delivery of islet transplants, due to substantial immediate destruction of transplanted islet mass (109). Robertson has raised the question of whether intraportal infusion is the most appropriate site for islet transplantation (110). Perhaps alternative sites including the omental pouch could be better suited for islet implantation, but modifications to improve vascularity and engraftment will be needed before such approaches are tested in the clinic.

1.6.3 Islet numbers in the pancreas

With all the technological advances in islet isolation over the last decade, islet yield has improved from 250,000 IEQ to 500,000 IEQ, a number which represents approximately half the theoretical IEQ in a normal pancreas (38, 100, 111-116). Efforts to minimize islet loss in the immediate post-transplant period will have a major impact on the clinical outcome (109). The median wait times for islet transplantation at Edmonton have increased substantially as the program continues to develop and grow. This underscores the lack of adequate supply of clinical grade islets for transplantation, and provides additional impetus to explore alternative approaches including use of growth factors and living donor islet transplantation to further meet potential demand.

1.6.4 Recent progress in single donor islet transplantation

Hering and colleagues in Minneapolis have recently reported remarkable success with single donor pancreas infusions leading to insulin independence (58, 117) Contributions to improved engraftment came from several innovative strategies, including the use of an anti-TNF alpha soluble Fc receptor, Etanercept, together with potent T cell depleting inductive immunosuppression and use of iodinanol rather than Ficoll® gradients for islet purification. Clearly the quality of the initial pancreas and the careful selection of recipients without high insulin requirements may also have contributed in part to this remarkable success (118).

1.6.5 Islet protection & regeneration

Pre-treating donors with anti-inflammatory and anti-apoptotic compounds such as 17β-estradiol or atorvastatin could potentially mitigate the negative impact of islet damage induced by brain-injury derived pro-inflammatory cytokines (119, 120). Immune depletion of donor dendritic cells by donor pre-treatment (121) with agents such as alemtuzumab may also enhance islet survival after transplantation by reducing immune sensitization. Incorporation with the anti-thrombotic (anti-IBMIR) strategies developed by Korsgren and colleagues using nicotinamide, inactivated factor VIIa or low-molecular weight dextran sulphate during islet culture or in the recipient post-transplant to inhibit islet tissue factor expression, will further enhance the success of the living donor approach (122, 123).

1.6.6 Alternative islet sources

Alternative sources of insulin secreting cells have been proposed to meet the demand for human islets in β cell replacement therapy (124). Xenogeneic porcine islets may offer an abundant source for islet transplantation. However hurdles that must be overcome include, 1) α-1,3-gal antigen which is present in most non-human primates, 2) zoonotic infections (including pig endogenous retroviruses (PERV), and 3) the potential stigma associated with xenotransplantation (125-127). Breeding and genetic modification of pigs to eliminate major xeno-antigens show promise in non-human primate models of islet transplantation (128).

Islet stem cells and genetic engineering of islet tissue may improve engraftment and function in vivo (129, 130). One strategy is to promote differentiation of pancreatic islet progenitor and ductal cells into insulin secreting cells (131). Another approach is to genetically engineer already differentiated cells such as adult (132, 133) or fetal (134) hepatocytes into insulin-producing cells. Murine studies are promising and show that the differentiated fetal hepatocytes can function *in vivo (135)*. Stem cells may be a potential sustainable source of insulin-producing cells in the future.

1.6.7 Living donor islet transplantation

Living donor islet transplantation may provide a unique opportunity to treat many more patients with unstable forms of T1DM. Despite remarkable success in clinical islet transplantation over the past 5 years, islet supply and metabolic function viability remain limiting factors when islets are derived from cadaveric organ donors (119). Living donors allow procurement of islets under ideal conditions, with closer HLA matching between donor and recipient.

Augmentation of the islet mass of the donor both in the months before and in the recovery phase after surgery, during islet culture, and subsequently in the islet recipient using combination growth factors (including GLP-1, exendin-4, EGF, gastrin, INGAP or hepatocyte growth factor), could further minimize the potential risk of diabetes in the donor, and could substantially enhance the rate of single donor islet transplant success in the recipient (136-140).

The first successful living donor islet transplant was carried out on January 19, 2005 in Kyoto, Japan in a collaboration between the Japanese and Edmonton programs (141). The first recipient remains insulin free with excellent glycemic control with 8 months of follow-up, and the living donor remains healthy with no complications and normal glucose control at this time. Recent developments in surgical technique, including the potential for laparoscopic or hand-assisted retrieval, may enhance the acceptability of the approach. Living donor islet transplantation may become applicable to children with T1DM and contribute to the reduction in the shortfall between supply and demand. It remains to be seen whether islets derived from living related donors (more closely HLA matched) will be less susceptible to acute rejection events, or perhaps more susceptible to autoimmune recurrence injury over time.

1.6.8 Procedural risks & complications

Currently portal access for islet delivery is achieved by either cannulation at time of surgical laparotomy or radiological percutaneous approach to the portal vein using fluoroscopic and ultrasound guidance. Procedure related risks of islet transplantation include bleeding, thrombosis, biliary puncture, discomfort, and transient rise in serum transaminase, and arteriovenous fistulae.

The surgical approach is associated with the risks common to any surgical procedures, including bleeding, infection, and anesthetic risk. The radiological procedure has the potential risk of bleeding once the catheter has been withdrawn from the liver track. Bleeding after islet implantation may be avoided if the catheter track is sealed along its entire length, effective approaches include the use of D-STAT by the Miami group, the combination of coils and gelfoam by the Minnesota group, and the use of microfibrillary collagen (Avitene® paste, made up in radiological contrast agent) by the Kyoto and Edmonton groups.

1.6.9 Long-term outcome of islet transplantation

The decline in rates of insulin independence observed in long-term follow-up with the Edmonton Protocol represents a significant challenge and unique opportunity to further understand the principles that promote and sustain islet survival. The Edmonton group has begun an extensive prospective investigation of factors that are likely influencing the fall in islet function in patients, and anticipate that the results will collectively provide instructive information over time. The study will look at: 1) serial islet graft biopsies of the liver in recipients at one year post-transplant then repeated at times of islet dysfunction; 2) serological analysis of donor HLA-related sensitization (panel reactive antibody (PRA) testing and data on donor-recipient cross-matching to one or multiple donors); 3) cytotoxic cytokine gene activity (granzyme B) monitoring pre- and at defined times post-transplant, and at additional time-points when there is evidence of islet dysfunction, in a collaboration with the University of Miami (142); 4) and detection of changes in autoantibody status (islet specific auto-antibodies (ICA512, GAD, and mIAA)). Other potential for causes of progressive islet dysfunction include: 1) chronic allograft rejection or undiagnosed acute rejection; 2) local islet toxicity from chronic exposure to tacrolimus, sirolimus, and other diabetogenic drugs; 3) recurrent autoimmunity; and/or 4) failure of islet regeneration (failure of cytokeratin-19 positive ductal cell element transdifferentiation to β cells) resulting from the anti-proliferative properties of sirolimus (101); 5) insulin resistance caused by receptor degradation; and 6) islet exhaustion through metabolic burn-out over time (as islets must secrete insulin at maximal capacity with no reserve or rest, distinct from the normal pancreas, where only about 10% of the total islet mass is functional at any one time to ensure glucose homeostasis).

1.6.10 Novel pharmacologic compounds in islet transplantation

A number of exciting, emerging compounds with distinct mechanisms of action will shortly be entering pilot clinical islet transplant trials. These agents provide an opportunity to develop less diabetogenic approaches with fewer non-immune related side-effects. Emerging opportunities include: 1) potent co-stimulatory blockade with belatacept (LEA29Y) is highly effective in promoting islet survival in primates and will be evaluated in Emory and Edmonton (143); 2) the lymphocyte homing agent FTY720 has be highly effective in controlling autoimmunity in NOD mice and in promoting marginal mass islet transplants in primates, and will be evaluated in Miami, Minnesota and Edmonton (144, 145); 3) the combination of anti-thymocyte globulin (ATG) and rituxumab (anti-CD20) induces tolerance to islet allografts in primates, and will be explored clinically by Naji and colleagues at the University of Pennsylvania; 4) the non-FcR-binding hOKT3-γ1-ala-ala antibody developed by Bluestone's group has been effective in abrogating autoimmunity in new-onset diabetes, and has facilitated single-donor islet transplant success in ongoing trials at the University of Minnesota (58, 146); 5) the T-cell depleting antibody alemtuzumab (Campath-1H) has shown promise in clinical solid organ transplantation, and is currently being evaluated in Edmonton and Miami, and 6) a potent, diphtheria-conjugated anti-CD3 immunotoxin combined with deoxyspergualin has provided impressive results with robust tolerance induced and sustained in islet transplants for over five years in a primate series treated by Thomas and colleagues at the University of Alabama (147). If these agents can match or exceed protection against both allo- and autoimmunity, and if the safety profiles prove to be superior to current therapies, islet transplantation will likely evolve even more dramatically in the near future.

1.7 THE FUTURE OF ISLET TRANSPLANTATION

More than fifteen years after insulin independence was first achieved in a patient with T1DM receiving an islet transplant, major challenges still remain (25). The decay of islet function over time indicates that there are many opportunities for improvement. More effective treatments are needed to control both acute rejection and recurrent autoimmunity. Improved control of HbA_{1c} and reduced risk of recurrent hypoglycemia are benefits of islet transplantation despite the status of insulin independence. The operative risk associated with islet transplantation may be less than those observed with whole pancreas strategies. However, prolonged insulin independence with whole pancreas transplantation and vastly improved surgical outcomes make whole organ transplantation a reasonable alternative for patients with T1DM with sufficient physiologic reserve. Current transplantation requires lifelong immunosuppression, and is limited to patients with recurrent severe hypoglycemia and severe labile diabetes. Remarkable opportunities lie ahead for the development of successful living donor islet transplantation, improved engraftment, islet proliferation *in vitro* and in the recipient, and novel tolerizing drugs with minimal side effects. Although life-long immunosuppression limits 'islet-alone' transplantation, islet transplantation in conjunction with previous or simultaneous solid organ transplant may be a viable option for patients with diabetes already receiving immunosuppression. Given these opportunities, it will be possible to expand the application of islet-alone transplantation beyond the most unstable forms of T1DM to include more patients including children in the future.

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CHAPTER TWO

COINHIBITORY T CELL SIGNALING IN ISLET ALLOGRAFT REJECTION AND TOLERANCE

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2.1 OVERVIEW

Auto-aggressive T cells directed against insulin secreting pancreatic β-cells mediate the development of type 1 diabetes mellitus. Islet transplantation offers superior glycemic control over exogenous insulin, but chronic immunosuppression limits its broad application. Pathogenic T cells are also important in allograft rejection. Inducing and maintaining antigen-specific peripheral T cell tolerance toward β-cells is an attractive strategy to prevent autoimmune disease, and to facilitate treatment of diabetes with islet allografts without long-term immunosuppression. Recent efforts have focused on blocking co-stimulatory T cell signals for tolerance induction. Although, co-stimulatory blockade can prolong graft survival, true immunological tolerance remains elusive. Co-stimulatory signals may even be required for the maintenance of peripheral tolerance. The discovery of novel co-inhibitory T cell pathways including CTLA4, PD-1, and BTLA, offers an alternative approach. Stimulating negative T cell co-signals alone or in combination may help induce tolerance. The focus of this review is to summarize the strategies directed at turning off the immune response by exploiting these negative co-signaling pathways in tolerance induction in islet transplantation. Activating several co-inhibitory pathways together may be synergistic in preventing pathogenic T cell responses. Tolerance induction will likely rely on understanding the balance of positive and negative signals affecting the state of T cell activation.

2.2 INTRODUCTION

Type 1 diabetes mellitus, a common endocrine disease, results from auto-aggressive T cells targeting insulin-producing β-cells within the islets of Langerhans in the pancreas. The selective and progressive destruction of the β-cells leads to insulin insufficiency and hyperglycemia. Current medical treatment for the majority of patients with type 1 diabetes consists of daily blood glucose monitoring and insulin injections. Although insulin therapy prolongs life by preventing lethal ketoacidosis, it does not prevent secondary complications such as proliferative retinopathy, peripheral neuropathy, and renal failure (1). Despite advances in insulin administration regimens, intensive insulin treatment can result in fatal hypoglycemia and remains a constant threat for a sub-group of patients with unstable diabetes. β-cell replacement by islet transplantation offers superior glycemic control for diabetes mellitus, and prevents or even reverses secondary complications, including nephropathy (2).

Ideally, the objective of clinical islet transplantation is to cure diabetes mellitus without the need for chronic immunosuppressive drug therapy (3). Although, immunosuppressive medications have made allograft transplantation possible, there are many potential side effects associated with their use, including risks of cancer and infection (4). Ironically, many of the immunosuppressive drugs needed to prevent graft rejection turn out to be diabetogenic. Corticosteroids cause insulin resistance, and cyclosporine (CsA) and tacrolimus (FK506) are implicated in decreased insulin secretion and impaired insulin sensitivity (5). Islet transplantation provides a unique opportunity to study allograft rejection and tolerance, because graft failure may mean a return to insulin dependence but does not result in certain death as in the case of a life-sustaining organ transplant. With the introduction of the 'Edmonton Protocol', a steroid-free, sirolimus-based, anti-rejection protocol for islet transplantation, the possibility of successful long-term clinical islet graft survival seems more attainable (6). However, for islet transplantation to be successful, both allo- and autoimmunity in allograft rejection must be overcome, either through the use of chronic immunosuppression or through induction of stable central or peripheral T cell tolerance. The mechanisms for inducing antigen-specific peripheral tolerance will rely on understanding the balance of signals affecting the state of T cell activation.

2.3 BALANCE OF CO-SIGNALS IN T CELL ACTIVATION

T cell activation and clonal expansion are critical in determining the outcome of the immune response to an allograft. Multiple signals regulate the differentiation and activation of T cells, including antigen stimulation, co-signaling, and cytokines. In the two-signal hypothesis, described in 1975 (7), a naïve T cell receives signal one through engagement of the T cell receptor (TCR) after encounter with antigenic peptide presented on major histocompatibility complex (MHC) molecules and receives signal two through co-stimulation (FIGURE 2-1a). Activated T cells secrete growth factors and cytokines, which act in an autocrine and paracrine fashion to drive proliferation, differentiation, and effector function. Signal one provides antigenic specificity, but signal two is required for optimal T cell activation. Signal two is dependent on signal one, and engagement of co-stimulatory receptors in the absence of the TCR signal does not induce a T cell response.

The two-signal theory explains why the absence of co-stimulatory signals may lead to decreased responses or, in some cases, induce anergy or tolerance (8). The idea of co-signaling molecules in lymphocyte activation was first described as part of the 'tripartite inactivation model' in 1971 (9), and expanded upon in subsequent years to include co-stimulators (10) and co-inhibitors (11). Discovery of new co-inhibitory pathways adds an additional dimension to the two-signal hypothesis, and suggests that a balance of complimentary positive and negative co-signals directs the antigen-specific T cell response towards activation or inhibition (12), rather than simply a lack of co-stimulatory signals (FIGURE 2-1b). Although co-stimulatory molecules have been well studied and described, the role of novel co-inhibitory molecules and their importance in regulating T cell immunity is only now becoming widely apparent (13).

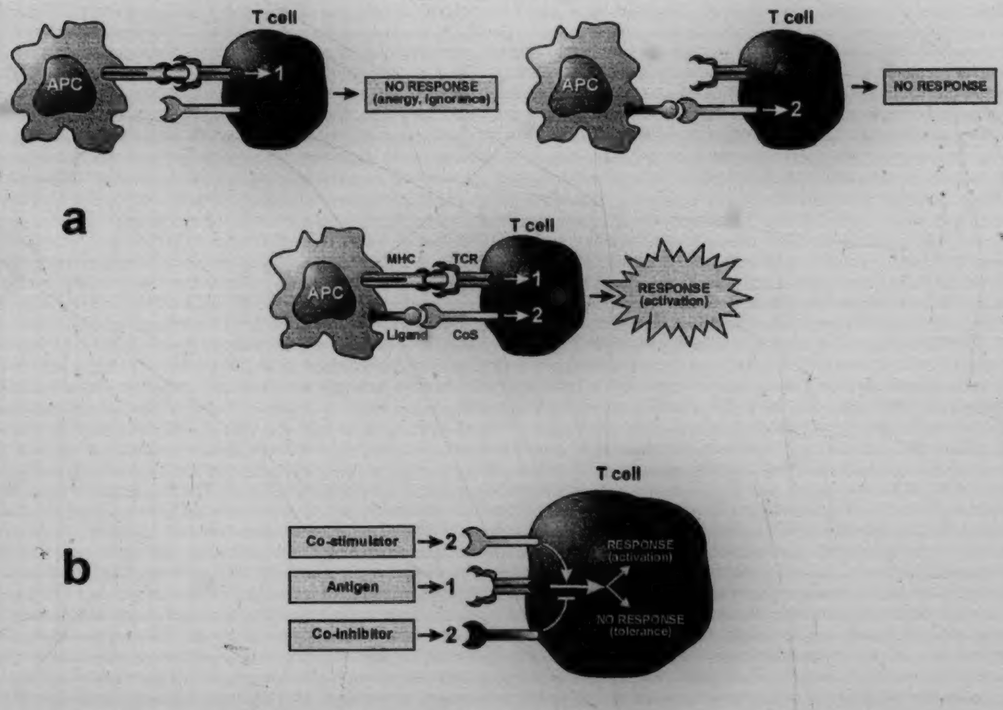
T cell
APC
1
NO RESPONSE (anergy, ignorance)
T cell
APC
2
NO RESPONSE
a
T cell
MHC
TCR
APC
1
2
Ligand
CoS
RESPONSE (activation)
T cell
b
Co-stimulator
2
Antigen
1
Co-inhibitor
2
RESPONSE (activation)
NO RESPONSE (tolerance)

Figure 2-1 The Co-signaling Models of T cell Activation (adapted from Chen(13))
(a) The Two-signal Model of T cell Activation (7): T cells receive an antigen specific **signal one** from T cell receptor (TCR) interaction with major histocompatibility complex (MHC) molecules presenting antigenic peptide on the surface of antigen presenting cells (APC). With no other signal, optimal activation does not occur and may even induce tolerance, anergy or immunological ignorance. **Signal two** depends on signal one, because co-stimulatory receptor (CoS) binding to its ligand alone does not induce a T cell response. Combination of the two signals is required for optimal activation of T cells. **(b) The Balance of Positive and Negative Co-signals in the Regulation of T cell Activation (11):** The TCR signal is essential for a T cell response, however, it does not determine activation or inhibition. Co-signaling molecules can be co-stimulatory or co-inhibitory and the ultimate fate of a T cell response depends on the balance between these opposing signals.

Four major types of inhibitory receptors have been described in lymphocytes, including target recognition of: 1) MHC class I, 2) the Fc portion of IgG, 3) various B7 family members, or most recently 4) one or more tumor necrosis factor (TNF) receptor family members (FIGURE 2-2) (14). The most recent TNFR-recognizing molecule identified is B and T lymphocyte attenuator (BTLA) (15). BTLA may be more closely related to KIR receptors found on NK cells than to PD-1 or CTLA-4. However, B7-recognizing inhibitory receptors along with BTLA belong to the immunoglobulin (Ig)-superfamily and appear to be the most promising targets in allograft tolerance and are the focus of this review.

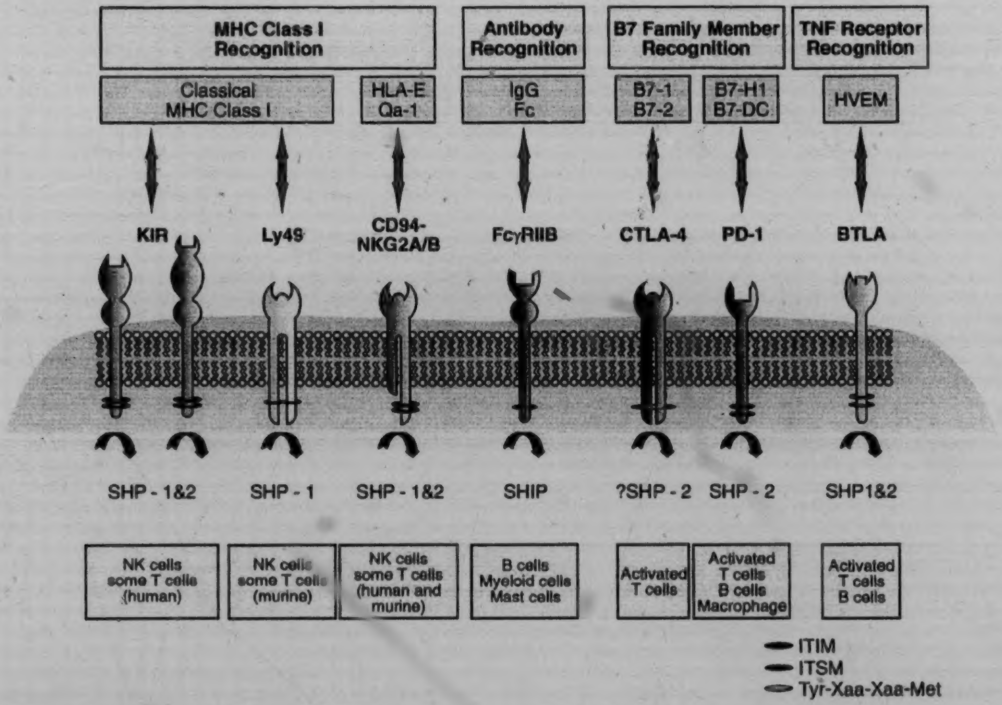
MHC Class I Recognition
Antibody Recognition
B7 Family Member Recognition
TNF Receptor Recognition
Classical MHC Class I
HLA-E Qa-1
IgG Fc
B7-1 B7-2
B7-H1 B7-DC
HVEM
KIR
Ly49
CD94-NKG2A/B
FcγRIIB
CTLA-4
PD-1
BTLA
SHP - 1&2
SHP - 1
SHP - 1&2
SHIP
?SHP - 2
SHP - 2
SHP1&2
NK cells some T cells (human)
NK cells some T cells (murine)
NK cells some T cells (human and murine)
B cells Myeloid cells Mast cells
Activated T cells
Activated T cells B cells Macrophage
Activated T cells B cells
ITIM
ITSM
Tyr-Xaa-Xaa-Met

Figure 2-2 Major Inhibitory Receptors Regulating Lymphocyte Activation (adapted from Leibson(14))
Inhibitory receptors recognizing either MHC class I, the Fc portion of IgG, different B7 family members, or tumor necrosis factor (TNF) receptor family members have been identified. Lymphocyte activation through ITAM-containing activating receptors can be blocked or attenuated by these inhibitory receptors when they bind with their respective ligands. When the inhibitory receptors are cross-linked, activator receptor induced phosphorylation of ITIM motifs in the cytoplasmic tails of most receptors results in interaction with SHP-1, SHP-2, or SHIP phosphatases, which act to dampen or completely eliminate T cell activation.

All three major types of transmembrane negative regulators: inhibitory MHC-recognizing receptors; inhibitory Fc-recognizing receptors; and, inhibitory receptors of the Ig-superfamily generate their inhibitory signals by a similar mechanism. Ligand binding of an activating receptor in combination with inhibitory receptor cross-linking induces phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic tails of most of these negative receptors, resulting in a signal cascade involving the recruitment of phosphatases including Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-1 (SHP-1), SHP-2, or SH2 domain-containing inositol-5 phosphatase (SHIP), which attenuate or block positive signals that would otherwise precipitate lymphocyte activation. The MHC-recognizing inhibitory receptors expressed on natural killer (NK) cells and subpopulations of activated T cells (a subset of cytotoxic T lymphocytes (CTL)), are capable of preventing cytotoxic function in these cells by blocking actin cytoskeleton-dependent formation of the immunological synapse and polarization of lipid rafts, which are essential for lymphocyte activation (16). Inhibitory Fc-recognizing receptor activation on mast cells, macrophages, and B cells ultimately blocks intracellular calcium signaling and regulates the threshold for activation and ongoing responses (17). The identified Ig-superfamily members, of which include inhibitory B7-recognizing receptors (cytotoxic T lymphocyte-associated antigen-4 (CTLA-4, CD152), programmed death-1 (PD-1)), and TNFR-recognizing inhibitory receptor BTLA, are expressed on activated lymphocytes, and thus regulate ongoing immune responses in lymphoid tissue and the periphery.

When T cells encounter self-antigen, or an innocuous foreign antigen, the absence of co-stimulatory signals (18, 19) or the presence of co-inhibitory signals prevents activation and the generation of large amounts of damaging immune products (11). The T cell may even become inactivated (anergy) or eliminated (apoptosis). Increasing evidence suggests it is the balance between positive and negative co-signaling that ultimately directs T cells towards allograft tolerance or rejection. Therefore, these co-signaling receptors provide appealing targets for strategies aimed at limiting the immune response in allograft rejection.

2.4 ROLE OF T CELLS IN ALLOGRAFT REJECTION

T cells play a central role in allograft rejection. Studies in the 1950s showed adoptive transfer of lymphocytes, but not serum antibody, conferred allograft immunity (20, 21). Subsequent work confirmed that T cells are necessary to transfer allograft rejection. In one study, monoclonal antibodies (mAb) to CD4+ or CD8+ T cells were injected into mice to deplete one or both subpopulations of T cells (22). These experiments suggested that CD4+ and CD8+ T cell subpopulations synergistically regulate allograft rejection.

T cells recognize antigen by several distinct pathways (FIGURE 2-3) (23). Through a direct pathway, recipient T cells identify allogeneic MHC class I, and II molecules expressed on donor cells as foreign molecules that resemble self-MHC plus immunogenic peptide. By an indirect pathway, recipient antigen-presenting cells (APC) internalize, and process donor antigen (i.e. donor MHC) and present them to T cells as foreign peptides on self-MHC class I, or II molecules. A third type of allorecognition called the semidirect pathway may result from intercellular transfer of MHC class I and II molecules (24). Recipient APCs could acquire allogeneic MHC class I molecules from donor APCs, and stimulate direct pathway CD8+ T cells along with stimulating indirect pathway CD4+ T cells after processing donor antigen.

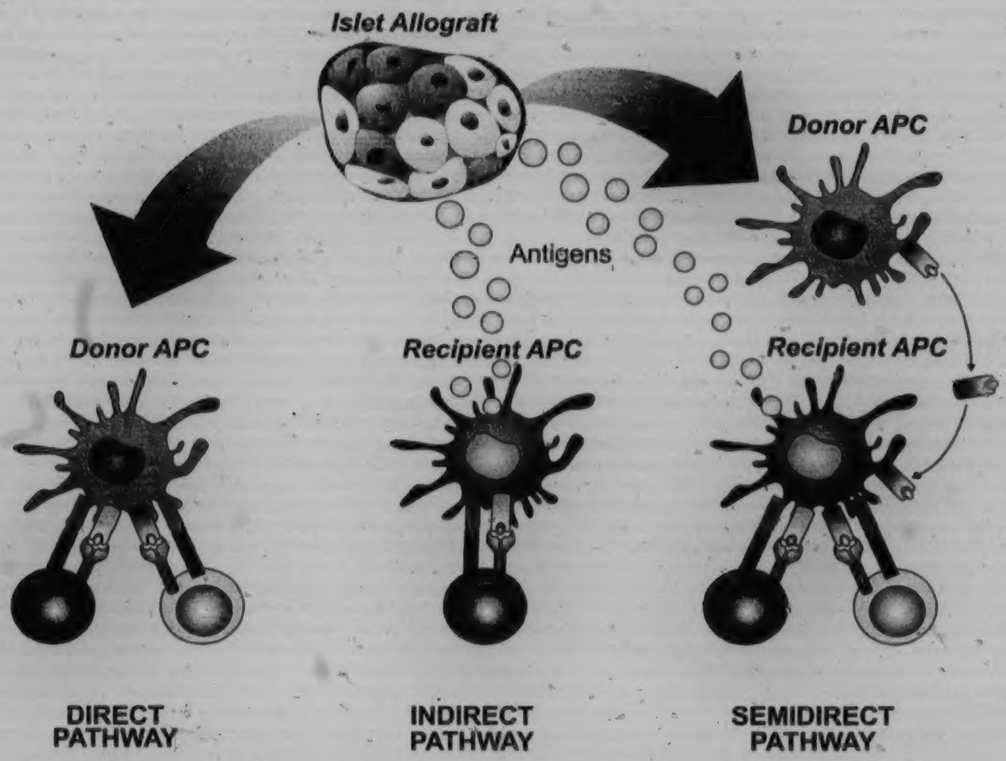
Islet Allograft
Donor APC
Antigens
Donor APC
Recipient APC
Recipient APC
DIRECT PATHWAY
INDIRECT PATHWAY
SEMIDIRECT PATHWAY

Figure 2-3 Direct, indirect, and semi-direct pathways of antigen recognition in islet allograft rejection

With appropriate co-stimulation, CD4+ helper T cells produce cytokines that promote the growth and activation of CD4+ T helper 1 (Th1) and CD8+ CTLs, macrophages, and B cells, which cause acute graft rejection by membrane damage and lysis of donor cells, delayed-type hypersensitivity, and antibody production, respectively (25). Antibody production by B cells does not play a major role in acute rejection in naive recipients, but antibodies produced after sensitization to donor cells cause antibody dependent cell-mediated cytotoxicity (ADCC), a complement-mediated response in hyperacute and accelerated rejection. Chronic rejection depends on both humoral and delayed-type hypersensitivity responses (26). The T cell plays a primary role in allograft rejection and therefore is a logical target for the development of strategies to induce tolerance.

2.5 PATHOLOGIC AND REGULATORY T CELLS IN AUTOIMMUNITY

Robust islet allograft function post-transplant requires control of both allo- and autoimmunity. Autoreactive T cells, which have escaped central and peripheral tolerance, mediate β-cell destruction in the non-obese diabetic (NOD) mice, a well-established model of type 1 diabetes (27, 28). Two major co-stimulatory pathways are implicated in these pathogenic T cell responses: CD28-B7 and CD40 ligand-CD40 (CD40L-CD40) pathways. Treatment of NOD mice with anti-B7-2 mAb decreases the incidence of diabetes (29), and NOD mice deficient for B7-2 do not develop diabetes (30). Similarly, NOD mice treated with anti-CD40L mAb (31) and CD40L-/- NOD mice (32, 33) are protected from diabetes.

The balance between auto-reactivity and immune regulation in the periphery determines whether NOD mice develop diabetes (34-36). Regulatory T cells (T-reg) play a central role in immune homeostasis (37, 38) and control of autoimmunity in the periphery (39, 40). Forkhead box protein 3 (Foxp3), a forkhead-winged helix family transcription factor expressed within murine CD4+ CD25+ T-regs, is as 'master gene' controlling the development and function of T-regs (41-43). T-regs are refractory to conventional T cell stimulation (44-46), and suppress adjacent CD4+ and CD8+ T cells through TCR plus CD28 co-stimulation dependent direct contact (44, 46, 47). Exogenous IL-2, or exposure to a lymphopenic environment or DC can break normal CD4+ CD25+ T-reg cell anergy (44, 45, 48, 49).

T-regs confer protection against autoimmune diabetes (34). NOD mice have decreased numbers of T-regs (50). CD28 co-stimulation is required for T-reg function (34, 51). Blocking the CD28-B7 pathway in NOD mice decreases activation of pathogenic T cells, but also affects T-reg cell development and in fact exacerbates diabetes (34, 52). Although, B7-2 is necessary for the optimal activation of pathogenic T cells in NOD mice, it is not required for the development of functional T-reg cells (53). Complete disruption of the CD28 co-signal or depletion of CD4+ CD25+ T cells allows pathogenic T cell activation and development of diabetes (53). Similarly, NOD mice deficient in both CD40L and CD28 showed decreased T-regs, and increased insulitis and diabetes as compared with NOD mice deficient in CD40L alone (53). Therefore, interfering with co-stimulatory signals may prevent normal regulatory T cells from working properly in the periphery, leading to paradoxical exacerbation of diabetes. Enhancing co-inhibitory molecules may avoid this potential complication.

Inducing tolerance in NOD mice is difficult because once T cells are activated by autoimmune mechanisms they become less dependent on co-stimulation for ongoing proliferation and cytokine production (54, 55). Prevention of type 1 diabetes using co-stimulatory blockade therapies are successful prior to the onset of insulitis, but stopping or reversing type 1 diabetes after auto-aggressive T cells are activated has so far been ineffective. A notable exception has been the remarkable recent findings of Strom and colleague who showed that hyperglycemic NOD mice can be restored to normality with combined treatment with IL-2 agonist, IL-15 antagonist and sirolimus (56). Some co-inhibitory molecules are expressed constitutively on CD4+CD25+ regulatory T cells (57), and may regulate and suppress ongoing autoimmunity, suggesting that manipulation of negative, in addition to positive, co-signals may be necessary for induction of tolerance in the case of autoimmune type 1 diabetes mellitus. After binding to different B7 and TNFR family members, the three identified Ig-superfamily inhibitory co-receptors CTLA-4, PD-1, and BTLA, inhibit immune activation as part of an integrated system of positive and negative signals that differentially influence naïve and activated cells (FIGURE 2-4).

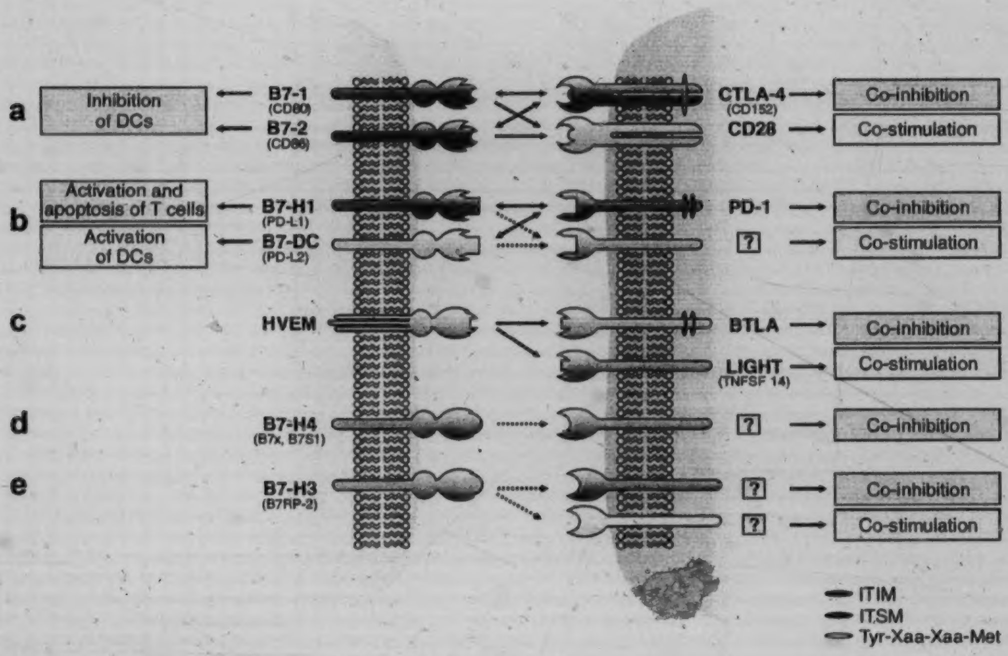
a
Inhibition of DCs
B7-1
(CD80)
B7-2
(CD86)
CTLA-4
(CD152)
CD28
Co-inhibition
Co-stimulation
b
Activation and apoptosis of T cells
Activation of DCs
B7-H1
(PD-L1)
B7-DC
(PD-L2)
PD-1
?
Co-inhibition
Co-stimulation
c
HVEM
BTLA
LIGHT
(TNFSF 14)
Co-inhibition
Co-stimulation
d
B7-H4
(B7x, B7S1)
?
Co-inhibition
e
B7-H3
(B7RP-2)
?
?
Co-inhibition
Co-stimulation
ITIM
ITSM
Tyr-Xaa-Xaa-Met

Figure 2-4 Co-inhibitory Pathways (adapted from Chen (13))
(a) CTLA-4: B7 Pathway: CD80 and CD86 regulate naïve T cell responses through co-stimulator CD28 and co-inhibitor CTLA-4. Reverse signaling can occur whereby CD80 and CD86 may act as negative receptors on dendritic cell (DC) (58). **(b) PD-1: PD-L Pathway:** PD-L1 and PD-L2 bind to PD-1 on lymphocytes to deliver a negative co-signal. The ligands PD-L1 and PD-L2 may also deliver a positive co-signal through an unidentified receptor (59). Reverse signaling delivered through PD-L1 on T cells can cause activation or apoptosis (60). Reverse signaling delivered through PD-L2 activates DC and thus enhances T cell activation (60, 61). **(c) BTLA: HVEM Pathway:** HVEM, recently identified as the ligand for BTLA (15), is currently a unique co-inhibitory molecules since it belongs to the TNFR family and also has a co-stimulatory receptor, LIGHT. **(d) ?: B7-H4 Pathway:** The receptor for B7-H4 is unknown, though evidence suggests it delivers a negative signal to T cells (62-64). **(e) ?: B7-H3 Pathway:** B7-H3 can reportedly deliver positive or negative signals, and its receptor(s) is still unknown (65, 66).

2.6 CTLA-4:B7 CO-INHIBITORY PATHWAY

The engagement of CD80 (B7-1) and CD86 (B7-2), by CD28 (67) and an inhibitory CTLA-4 receptor on T cells is the most extensively studied co-signaling pathway (68). Upon B7-1/B7-2 binding, constitutively expressed CD28 gives a strong co-stimulatory signal to naïve T cells. CTLA-4 expression is not constitutively expressed by naïve T cells but is induced, peaking at 48-72 hours after T cell stimulation (69). Binding of CTLA-4 by B7-1/B7-2 generates signals that inhibit cell cycle progression, proliferation and IL-2 synthesis (68). Hence, CTLA-4 limits the number and function of CD4+ and CD8+ cells in response to allogeneic challenge and can induce peripheral T cell tolerance or anergy (70).

CTLA-4 can be a very potent co-inhibitory receptor, and binds B7-1 with at least a one log higher affinity than CD28 (71), whereas B7-2 displays less bias (72). The CTLA-4 cytoplasmic region contains a Tyr-Xaa-Xaa-Met motif (Gly-Val-Tyr-Val-Lys-Met), which has an inhibitory function (73). The negative co-signaling function of CTLA-4 is supported by the observation that CTLA4-deficient mice spontaneously develop rapidly lethal lymphoproliferative disease with polyclonal T cell infiltration of multiple organ systems (TABLE 2-1) (74, 75).

Table 2-1 Co-inhibitory pathways and Autoimmunity

Model	Strain	Outcome	References
CTLA-4-/-	Murine	Spontaneously develop rapidly lethal lymphoproliferative disease with polyclonal T cell infiltration of multiple organ systems	(74, 75)
PD-1-/-	Murine C57BL/6 background	Develop splenomegaly, increased serum Ig, late onset immune-complex glomerulonephritis, and arthritis	(76)
PD-1-/-	Murine BALB/c background	Develop lymphocyte-dependent autoimmune dilated cardiomyopathy	(77, 78)
BTLA-/-	Murine	Display a subtle phenotype; enhanced sensitivity to EAE; Th1 cells show enhanced proliferation	(79)
BTLA-/-	Murine	Fully MHC-mismatched recipients show unexpected prolongation of survival of cardiac allografts; conversely, partially MHC-mismatched recipients show accelerated rejection.	(80)

Furthermore, administration of anti-CTLA-4 mAb exacerbates autoimmune disease (81, 82), and enhances anti-tumor immunity (83). In addition, subpopulations of regulatory CD4+CD25+ T cells can express CTLA-4 constitutively (57), and CTLA-4 can dampen immune responses by inducing the production of inhibitory cytokines such as transforming growth factor-β (84). CTLA-4 on T cells may also act as a ligand for B7-1 and B7-2 on dendritic cells (DC) (58). This reverse signal induces the expression of indoleamine 2,3-dioxygenase (IDO) in DC, promoting tryptophan catabolism that ultimately blunts T cell responses (58). Hence, CTLA-4 may be critically involved in several mechanisms essential for both suppressing spontaneous autoimmunity against self-antigens (as in autoimmune diabetes) and inhibiting activated T cells specific for foreign antigens. As CTLA-4 can inhibit both T cell and DC responses, it is an attractive target for promoting tolerance in islet transplantation, given that grafts have to contend with host allo- and autoimmune responses.

2.6.1 CTLA-4 in tolerance induction

Several hurdles limit the utility of CTLA-4 signaling as a therapeutic approach. Firstly, the CTLA-4 receptor is expressed by activated T cells but not most naïve T cells (69). Secondly, the recombinant fusion protein CTLA-4-immunoglobulin (CTLA4-Ig) containing the CTLA-4 extracellular domain fused to an IgG1 heavy chain (Fc) tail, induces long-term allograft survival by interfering with CD28:B7-1/B7-2 signaling and not by activating CTLA-4 negative co-signals (85), taking advantage of the fact that CTLA-4 has a higher affinity for B7-1/B7-2 than CD28. Thirdly, there are no soluble *in vivo* CTLA-4 cross-linking agents available at present. In fact, anti-CTLA-4 mAbs block the negative signal and augment rather than inhibit immune responses. An alternative strategy is to express an anti-CTLA-4 single chain Fv antibody (scFv) on the cell surface in a tumor cell line (86, 87). CTLA-4 cross-linking and co-ligation with the TCR in these cells down-regulates both CD4+ and CD8+ T cell activation. Another approach is to administer tolerogenic anti-CD45RB mAb, which rapidly enhances CTLA-4 expression on a subset of CD4+ T cells in the absence of T cell activation (88). When CsA is used in combination with anti-CD45RB mAb, acute rejection is precipitated, possibly due to downregulation of CTLA-4; anti-CD45RB mAb is unable to induce tolerance in mice deficient in CTLA-4, B7-1 or B7-2 (88). Despite these challenges, CTLA-4 has been studied extensively for potential tolerance induction in allotransplantation.

2.6.2 Targeting CTLA-4 in Transplantation

Targeting the CTLA-4 pathway for induction of tolerance has generally been undertaken using the fusion protein CTLA4-Ig, or its more potent derivative, LEA29Y. CTLA4-Ig prolongs survival of allografts and xenografts in various rodent models (89-91). Administration of CTLA4-Ig can induce antigen-specific tolerance in some but not all rodent models; in some cases, cardiac allografts survive indefinitely in recipients treated with CTLA4-Ig, and recipients accept skin grafts from the original donor, but reject third party skin grafts (91, 92).

In studies involving donor specific transfusion (DST) and anti-CD40L mAb therapy, the addition of CTLA4-Ig therapy prevented tolerance (93); and, tolerance induced by CTLA4-Ig combined with DST was prevented when anti-CTLA-4 mAb was administered concomitantly (85). These findings suggests that although CTLA4-Ig might primarily act by interfering with CD28/B7 signaling (given B7-1 markedly favors CTLA-4 engagement over CD28) (67), B7 interaction with CTLA-4 is essential for achieving tolerance (94, 95). Furthermore, 'double knockout' mice, deficient in B7-1 and B7-2, do not develop acute or chronic rejection of cardiac allografts (96). However, 'triple knockout' mice lacking CTLA-4, B7-1, and B7-2, do reject islet allografts (84). This is partially due to the expression of B7-1 and B7-2 by the islet graft. Although CTLA4-Ig can induce long-term engraftment despite the absence of CTLA-4 signaling in these mice, this may be due to ignorance rather than tolerance, because long-term survivors readily reject a second graft from the same donor strain. Therefore, indiscriminate use of CTLA4-Ig may hinder rather than promote the induction of tolerance by interfering with negative CTLA-4 signaling in addition to blocking positive CD28/B7 signaling (34, 97, 98).

Strategies aimed at enhancing CTLA-4 expression show that a short course of anti-CD45RB monotherapy prolongs the survival of rat islet xenografts in C57BL/6 recipient mice (99). Unfortunately, the tolerance induced by anti-CD45RB does not translate to the NOD mouse model (100). As with many other therapies, the NOD mice seem to be resistant to tolerance induction and reject islet allografts despite success in other strains.

CTLA4-Ig plus co-stimulatory blockade with anti-CD40L (CD154) mAb have synergistic effects on skin and cardiac (89), renal (101) and aortic (102) allograft survival. While LEA29Y therapy in non-human primate islet allograft recipients provided excellent and stable graft function when the agent was administered repeatedly, rejection occurred upon cessation of therapy (103). Despite the success of anti-CD40L mAb in rodent models, its clinical application is in question because of increased risk of thromboembolic events in clinical trials. To avoid this potential complication an antibody directed against CD40 was developed as an alternative. Use of anti-CD40 mAb (Chi220, BMS-224819) plus LEA29Y holds promise and allows long-term survival of islet allografts in non-human primates (104).

2.7 PD1:PDL CO-INHIBITORY PATHWAY

PD-1, a 55 kD glycoprotein, was originally discovered in a T cell hybridoma undergoing apoptosis (105). Subsequent studies did not support a role for PD-1 in apoptosis but rather showed its expression is induced on T and B cells upon activation (106). PD-1 is also constitutively expressed by immature thymocytes, NK cells, and macrophages (107). PD-1 expression is thereby more broadly distributed than CTLA-4, which is limited to T cells, suggesting the two co-inhibitory molecules may play complementary roles in maintaining peripheral tolerance (108). PD-1 contains an ITIM motif (109), as well as an immunoreceptor tyrosine-based switch motif (ITSM), in is cytoplasmic tail, which is conserved between mouse and human (110, 111). The ITIM and ITSM motifs have the potential to recruit SHP-2 to deliver an inhibitory signal (112). PD-1 has two ligands, PD-L1 (113) and PD-L2 (114), which are also known as B7-H1 (115), and B7-DC (116) respectively.

Initial evidence for the co-inhibitory role of PD-1 comes from observations in PD-1 knockout mice (TABLE 2-1). PD-1-deficient C57BL/6 mice develop splenomegaly, increased serum Ig, late onset immune-complex glomerulonephritis, and arthritis (76), whereas PD-1-deficient BALB/c mice develop autoimmune dilated cardiomyopathy (77, 78). Compared to CTLA-4-deficient mice, PD-1-deficient mice develop autoimmune disease that is both delayed (months) and decreased in severity. The roles of PD-L1 and PD-L2 are less consistent, and in some non-transplant contexts even display apparent co-stimulatory functions (59). Reverse signaling delivered through PD-L1 on T cells can cause activation as well as apoptosis (60); while signaling through PD-L2 activates DC and can enhance T cell activation (60, 61). The phenotype of PD-L1 deficient mice is normal except for CD8+ T cell accumulation in the liver (117), and PD-L2 deficient mice appear normal up to at least one year of age (118).

Inhibitory function of PD-1 is seen in NOD and other autoimmune mouse models. Targeting PD-1 in NOD mice caused earlier onset of diabetes (119). Studies suggest that PD-1 may be involved in the maintenance of self-tolerance in the periphery. Although an important issue arises that the sole anti-PD-1 mAb available for use in mice, J43, does not appear to block PD-1 interaction with its ligands; beneficial effects seen *in vivo* might therefore reflect depletion of PD-1+ cells. In NOD mice, anti-PD-L1 but not anti-PD-L2 mAb induced earlier onset of diabetes (119). Whether PD-L1 functions to enhance or deter islet allograft survival, may depend on the type of cell it is expressed on, e.g. parenchymal versus immune cell. Transgenic expression of PD-L1 on islet cells increased the incidence of diabetes, mediated by increased CD8+ T cell proliferation and promoted islet allograft rejection (120). In contrast, blocking the monocyte chemoattractant protein-1 (MCP-1)/CCR2 chemokine pathway induces permanent islet allograft survival through a PD-1/PD-L1 dependent mechanism (121). Intra-graft expression of MCP-1 and its receptor CCR2 is associated with islet graft rejection. Blocking MCP-1 signaling with a mAb combined with sub-therapeutic sirolimus prolongs islet allograft survival and correlates with mononuclear cell infiltrates expressing PD-1 and PD-L1, while anti-PD-L1 mAb abolishes this survival benefit. Hence, PD-L1 and PD-L2 demonstrate negative or positive co-signaling capabilities, which appear to vary depending upon the context and model under consideration.

Other autoimmune models show similar results. In experimental autoimmune encephalomyelitis (EAE) models, C57BL/6 mice developed worse disease when PD-1 was targeted using an anti-PD-1 antibody (122). In EAE, blocking PD-L1 had no effect but blocking PD-L2 worsened disease (122). Blocking PD-L1, but not PD-L2 signal, in a colitis model induced by transfer of CD4+CD45RBhigh T cells, caused suppression of the disease, decreased lymphocyte infiltration, and reduced Th1, but not T helper 2 (Th2), cytokines from T cells (123).

2.7.1 Targeting PD1 in Transplantation

Studies on the PD-1 pathway in allograft transplantation are few. Cardiac allograft recipients treated with PDL1-Ig as a PD-1 agonist rejected allografts at the same rate as controls. However, PDL1-Ig administration in CD28-deficient recipients or in conjunction with CsA in fully MHC-mismatched combinations prolonged cardiac allograft survival (124). PDL1-Ig combined with anti-CD154 also prevented chronic allograft rejection (124) and prolonged islet allograft survival (125). Thus in the context of T cell co-stimulatory CD28/CD154 blockade, or immunosuppression, targeting PD-1 can prevent allograft rejection and modulate T and B cell-dependent pathogenic immune responses *in vivo*.

2.8 BTLA:HVEM CO-INHIBITORY PATHWAY

BTLA (79), a newly described co-inhibitory molecule which is part of the Ig-superfamily, is expressed by B cells, small numbers of resting T cells, macrophages and bone marrow-derived myeloid DC (126). Upon T cell activation and differentiation, only Th1 cells express BTLA and receptor expression is independent of IL-12 or IFN-γ. BTLA has structural and functional similarities to CTLA-4 and PD-1, but has a broader expression than CTLA-4 (69), which is limited to T cells, and has a more restricted expression than PD-1, whose expression is detected on T, B and myeloid cells (108). BTLA has three cytoplasmic domain tyrosine residues (Y226, Y257, and Y282), which are contained in sequence motifs conserved across human and murine receptors (79). The first tyrosine is in a potential Grb2-binding site (127), and the other two are in ITIM motifs (128). Y282 appears to resemble the ITSM in PD-1 (76) and signaling lymphocytic activation molecule (SLAM) (129). BTLA also shares similarities with killer Ig-related (KIR) receptors on NK cells (14). The co-inhibitory function of BTLA is supported by several observations (79). Firstly, BTLA cross-linking, like PD-1, upon TCR ligation *in vitro* induces a signal that attenuates production of IL-2, and dampens both T cell activation and B cell proliferation. Secondly, BTLA-deficient mice have increased humoral responses, and develop worse EAE, indicating inhibitory effects of BTLA on B and T cell responses *in vivo*. Finally, Th1 cells show enhanced proliferation in BTLA-deficient mice relative to wild-type Th1 cells.

In earlier studies, the proposed ligand for BTLA was B7-H4 (62) (B7x (63), B7-S1 (64)) based on evidence that B7H4-Ig had minimal binding to BTLA-deficient lymphocytes compared to wild-type lymphocytes. This observation requires caution, because it is possible that the receptor for B7-H4 is downregulated in the absence of BTLA. Although mRNA encoding B7-H4 is detected widely in human tissue, it is not constitutively expressed in most cells (130). Its expression can be induced in a variety of cells including, T cells, B cells, monocytes and DC after *in vitro* stimulation. Administration of B7H4-Ig decreases antigen-specific T cell responses (13).

In mice, B7H4-Ig acts by inhibiting TCR-mediated proliferation and causes cell cycle arrest. It also inhibits the function of CD8+ cytotoxic T lymphocytes and prolongs survival in a graft-versus-host disease (GVHD) model (62). On the other hand, blocking B7-H4 with mAb promotes T cell proliferation and IL-2 production. *In vivo* treatment with anti-B7-H4 blocking mAb exacerbates EAE (64), supporting a co-inhibitory role for B7-H4.

However, there is no evidence for a direct binding between BTLA and B7-H4. In particular, experiments using tetramers and Fc fusion proteins of BTLA and B7-H4 mixed with transfected cells expressing each co-signaling molecule fail to show BTLA/B7-H4 interaction, and BTLA-Ig fusion protein binds to naïve T cells, which do not express B7-H4 (15). Recent evidence definitively illustrates that B7-H4 is not the ligand for BTLA; instead herpesvirus entry mediator (HVEM), a TNFR family member, was identified as the unique ligand for BTLA in mice (15). Both mouse and human BTLA-Ig fusion proteins selectively interact with mouse and human HVEM, respectively. In addition, mouse BTLA tetramers bind to mouse HVEM. The BTLA extracellular immunoglobulin domain binds to the most membrane-distant cysteine-rich domain (CRD-1) of HVEM, a site that is unique compared with that of the additional HVEM ligands, LIGHT (homologous to lymphotoxin, inducible expression, competing for GpD of herpes virus, that binds to the HVEM, and is expressed on activated T lymphocytes) (131) and lymphotoxin-α (LTα). HVEM binding induces BTLA intracellular tail tyrosine phosphorylation and inhibits T-cell proliferation (15). HVEM-BTLA is a novel pathway, which provides a co-inhibitory signal to T cells and may prevent T cell activation.

2.8.1 Targeting BTLA in Transplantation

BTLA-deficient mice display only a subtle phenotype, but data suggest that the BTLA co-inhibitory pathway has a non-redundant role in attenuation of B and T cell responses (TABLE 2-1) (132). There are no completed studies using the BTLA co-inhibitory pathway in islet allotransplantation, because anti-BTLA mAbs currently available block rather that enhance the negative co-signal. BTLA expression is up-regulated on CD4+ and CD8+ T cells undergoing alloactivation *in vitro* and *in vivo*, and rejecting cardiac allografts show increased intra-graft expression of BTLA mRNA and protein (80). However, fully MHC-mismatched cardiac allografts transplanted into BTLA-deficient mice have decreased T cell proliferation and cytokine production, and a doubling of graft survival, and comparable data were seen using a neutralizing anti-BTLA mAb. Additional benefits of blocking BTLA were also seen in allograft recipients treated with a sub-therapeutic course of sirolimus or co-stimulation blockade (CTLA4-Ig). These findings indicate, unexpectedly, that BTLA expression can enhance alloresponses. Conversely, partially MHC-mismatched cardiac allograft recipients deficient in BTLA or its ligand HVEM had accelerated rejection but the same grafts survived indefinitely in wild-type control recipients (80). The current working explanation for these surprising results is that up-regulation of PD-1 is more important than that of BTLA in the context of fully MHC-mismatched allografts, whereas partial MHC-mismatches induce negligible PD-1 expression by alloreactive T cells but strong BTLA up-regulation (80). As data concerning the novel BTLA/HVEM pathway unfolds, its role as a negative co-signaling molecule is becoming more complex and interesting, and much remains to be investigated.

2.9 B7-H3 PATHWAY

B7-H3 is a relatively new B7 family member that was initially described on human DC (133). mRNA encoding B7-H3 is found in lymphoid and non-lymphoid tissue (134). B7-H3 expression can be induced on monocytes by granulocyte-macrophage colony-stimulating factor (GM-CSF) and DCs by IFN-γ (133). Initial reports suggested B7-H3 could stimulate human CD4+ and CD8+ T cell proliferation and IFN-γ production (133, 134), but other reports suggested murine B7-H3 is a co-inhibitory molecule. Studies using B7-H3-deficient mice demonstrate that B7-H3 down-regulates Th1 responses (135). Soluble mouse B7-H3-Ig fusion protein reduces T cell proliferation and cytokine production, and blocks NFAT, NF-κB, and AP-1 transcriptional factors (65). Furthermore, the antibody blocking B7-H3 increases T cell proliferation and IL-2 secretion *in vitro*, and enhances EAE *in vivo* (135).

2.9.1 B7-H3 in Transplantation

In contrast, the first study to examine B7-H3 in allograft rejection and tolerance supports co-stimulatory function in T cell-mediated allograft responses (66). Sub-therapeutic sirolimus treatment in B7-H3-deficient mice prolonged cardiac and islet allograft survival indefinitely. In addition, B7-H3-deficient mice had decreased cytokine, chemokine, and chemokine receptor expression in cardiac allografts. Hence, the role of B7-H3 is controversial and is still being defined, but may be another potential target for tolerance induction.

2.10 FUTURE DIRECTION AND CONCLUSIONS

To achieve tolerance in islet transplantation for type I diabetes mellitus, both allo- and autoimmunity must be addressed. Co-stimulatory blockade may prevent activation of alloreactive T cells. However, interfering with co-stimulatory signals may also prevent normal regulatory T cell function, tipping the balance in favor of auto-reactive pathogenic T cells leading to exacerbation of autoimmune diabetes. Enhancing co-inhibitory molecules may avoid this potential complication. The discovery of negative co-signaling molecules such as CTLA-4, PD-1, and BTLA provides new targets for therapies in prevention of islet allograft rejection and for tolerance induction (TABLE 2-2).

However, there are several obstacles that must be overcome before these co-inhibitory pathways can be used. There are no soluble reagents currently available that will activate the negative co-signal CTLA-4, or BTLA. Even the efficacy of anti-PD-1 mAb (J43) in mice may reflect depletion of PD-1+ cells, rather than stimulation of the negative signal.

Available therapies targeting CTLA-4 rely on disrupting the co-stimulatory CD28 pathway, but may interfere with CTLA-4 co-inhibition itself. Indiscriminate use of CTLA4-Ig may hinder rather than promote the induction of tolerance. The roles of PD-L1 and PD-L2, ligands for PD-1, are not clear and they paradoxically exhibit activating function in some systems. Finally, BTLA, a novel member of the inhibitory receptors, may prove to be important in limiting the Th1 response. Recent studies indicate complex and unexpected interactions between co-inhibitory signals, and increasing cross-talk between positive and negative co-signaling molecules.

Strategies to date have focused on combining co-inhibitory signals in the context of suboptimal co-stimulation or immunosuppression. However, the mechanisms of action and patterns of expression on lymphocytes suggest that the three inhibitory receptors

Table 2-2 Strategies involving T cell co-inhibition in transplantation

Pathway	Reagent/Strategy	Outcome	Refs
CTLA-4 (CD28 co-stimulatory blockade)	CTLA4-Ig	Prolongs survival of pancreatic islet allografts and xenografts; benefit of human CTLA4-Ig on islet graft survival in nonhuman primates much less pronounced; cardiac allografts can survive indefinitely	(89-91, 136)
	CTLA4-Ig + DST	Long-term graft survival and donor-specific tolerance; survival prevented when anti-CTLA-4 is administered concomitantly	(85)
	CTLA4-Ig + anti-CD40L	Prolong skin and cardiac, renal, and aortic graft survival	(89, 101, 102)
	LEA29Y + anti-CD40	LEA29Y and anti-CD40 dramatically improved long-term islet allograft survival in nonhuman primate model	(104)
CTLA-4 (stimulation of co-inhibitor)	scFv against CTLA-4	Cross-linking and co-ligation with the T cell receptor (TCR) in these cells down-regulates CD4+ and CD8+ T cell activation	(86, 87)
CTLA-4 (up-regulation)	Anti-CD45RB	prolongs survival of rat islet xenografts in C57BL/6 mice	(88, 99)
PD-1 (Ligand over-expression)	Transgenic expression of PD-L1 on islets	Increases incidence of diabetes, mediated by increased CD8+ T cell proliferation	(120)
	Transplantation of PD-L1 transgenic islets	Increased rejection of islet graft	(120)
PD-1 (stimulation of co-inhibitor)	PDL1-Ig in CD28-deficient recipients or PDL1-Ig + CsA	Fully MHC-mismatched combinations prolonged cardiac allograft survival	(124)
PD-1 (stimulation of co-inhibitor)	PDL1-Ig + anti-CD154	Prolonged islet allograft survival	(125)
BTLA (Blocking co-inhibitor)	Anti-BTLA mAb	Prolongation of cardiac allografts survival in fully MHC-mismatched recipients but acceleration of rejection of partially MHC-mismatched allografts	(80)
BTLA (stimulation of co-inhibitor)	HVEM-Ig	Studies pending	
?	B7H4-Ig	Decreases antigen-specific T cell responses; inhibits TCR-mediated proliferation and causes cell cycle arrest; inhibits function of CD8+ CTL and prolongs survival in GVHD	(62, 63, 65)
?	B7H3-Ig	Reduces T cell proliferation and cytokine production; it also, blocks NFAT, NF-κB, and AP-1 transcriptional factors	(133)
?	B7-H3-/- (In contrast to above)	Sub-therapeutic sirolimus treatment in B7-H3-deficient mice prolonged cardiac and islet allograft survival indefinitely	(66)

serve complementary, non-redundant roles in attenuation of T cell immune responses. Therefore, combining activation of the three co-inhibitory molecules may be synergistic in the induction of islet transplantation tolerance. Many questions, however, remain to be answered. Future investigation of tolerance must also address the problem of recurrent autoimmune disease in type I diabetes. Further studies are required before targeting negative co-signaling can be translated to clinical islet transplantation. The primary focus of current investigation should be directed towards the development of appropriate reagents that stimulate these negative T cell co-signals to exploit the new co-inhibitory pathways on the road to islet allograft tolerance.

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CHAPTER THREE

HUMAN ISLET FUNCTION IS NOT IMPAIRED BY THE SPHINGOSINE-1-PHOSPATE RECEPTOR MODULATOR FTY720

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3.1 OVERVIEW

Clinical islet transplantation for type 1 diabetes mellitus currently requires potent immunosuppressive drugs, which limits the procedure to the most severe forms of the disease, and many of the drugs are directly β-cell toxic. A class of compounds called sphingosine-1-phosphate receptor modulators has been explored in transplantation and shown to be highly effective in multiple sclerosis and other autoimmune conditions. While FTY720, the first drug in this class, may not move forward initially in transplantation, this class requires detailed investigation to assess direct impact upon human β-cell function and survival. We set out to evaluate the effects of FTY720 on human islets *in vitro* by investigating glucose-stimulated insulin and apoptosis; and *in vivo*, after transplantation into immunodeficient mice with chemically induced diabetes, by examining blood glucose levels, oral glucose tolerance tests, and stimulated human C-peptide over a 50-day follow-up period. Our data showed that neither *in* vitro nor *in vivo* human islet function was impaired by FTY720 exposure. Since FTY720 demonstrated no detrimental effects on human islet function *in vitro* or *in vivo*, emerging S1PR modulators may prove to be useful adjuncts in clinical islet transplantation through lack of diabetogenicity and potent immunological protection.

3.2 INTRODUCTION

Despite recent advances in islet transplantation for type 1 diabetes mellitus (T1DM) (*1, 2*), the procedure still relies on toxic immunosuppressive agents. The search for potent immunomodulatory therapies that promote islet engraftment and function while preventing rejection and autoimmune recurrence are important current goals.

FTY720 is the first drug in a class of sphingosine-1-phosphate receptor (S1PR) modulators (3) that acts by down-regulating the expression of the $S1P_1$ receptors on lymphocytes, thereby sequestering them to secondary lymphoid tissue while markedly reducing circulating lymphocytes in the periphery and transplant grafts (4-8). Fu *et al* demonstrated that continuous FTY720 was potent in preventing both autoimmune diabetes recurrence and islet allograft rejection in non-obese diabetic (NOD) mice (5). Wijkstrom *et al* showed prolonged islet allograft survival and optimal function of marginal mass islet grafts in primates (9). Other compounds in the S1PR modulator class are currently in development.

S1PRs are expressed on isolated murine islets and extracellular engagement with their natural ligand sphingosine-1-phosphate (S1P) induces a dose dependent reduction in glucagon-like peptide-1 (GLP-1)-stimulated insulin secretion (10). In addition, S1P acts as an intracellular second messenger (11), and endogenous β-cell sphingosine kinases have been reported in isolated murine islets *in vitro* (12). Therefore FTY720 phosphorylation to FTY720-P may have potentially adverse extracellular and intracellular effects similar to S1P on islet survival and function. *In vitro* studies of mouse islets exposed to FTY720 suggest that it does not impair function (5). We herein further evaluate the potential impact of FTY720 on isolated human islets *in vitro* and *in vivo* after transplantation into immunodeficient mice with chemically induced diabetes.

3.3 MATERIALS AND METHODS

3.3.1 *Human islet isolation and culture*

Human pancreases were recovered from deceased donors with prior informed written consent. Islets were isolated from the whole pancreas using a mechanical and enzymatic dissociation process that has been previously described (13). Islets were cultured for 48 hours for *in vitro* testing and for 3 to 4 days for *in vivo* transplantation studies, with daily medium changes.

3.3.2 *FTY720 preparation and administration*

FTY720 was generously supplied by Novartis (Novartis, Basel, Switzerland). FTY720 is sparingly soluble in aqueous solution, and therefore was dissolved in ethanol and diluted 1:1 with distilled water. Human islets were incubated in culture for 48 hours at 22°C, 5% CO_2, in the presence of either no additives ('control'), 1:1 ethanol:distilled water ('vehicle'), 'physiological' FTY720 (10ng/ml), or 'high' FTY720 (1000ng/ml). For *in vivo* transplantation studies, FTY720 was administered daily at 1mg/kg/day by oral gavage.

3.3.3 *Glucose-stimulated insulin release assay*

For each group, duplicate aliquots of ~500 human islet equivalents (IEQ) were washed and incubated in 5ml 'low-glucose' medium at 37°C and 5% CO_2 for 10 minutes. Then the medium was replaced with either 2ml of 'low-' (2.8mM) or 'high-glucose' (20.0mM) medium (RPMI-1640 L-glutamine supplemented with 2mM 3-isobutyl-1-methylxanthine IBMX, 2.5% penicillin/streptomycin, 5% BSA fraction V, and 0.2% sodium bicarbonate; Sigma-Aldrich, Oakville, ON, Canada) and transferred to a non-tissue culture treated 6 well plate for a 2 hour static assay at 37°C and 5% CO_2. Special

Investigations Laboratory at the University of Alberta analyzed the supernatant for human insulin by ELISA (Roche Diagnostics, Indianapolis, IN, USA). The insulin assay had a lower detection limit of 0.200μU/ml (1.39pmol/L). The stimulation index (SI) was calculated by dividing insulin concentration in high-glucose by insulin in low-glucose.

3.3.4 TUNEL apoptosis assay

Apoptosis in human islets was quantified using TdT-mediated dUTP nick-end labeling (TUNEL) staining. Cultured human islets were fixed in 4% paraformaldehyde, embedded in agar, and processed in paraffin to obtain 10 μm sections. Human β-cells were identified by insulin staining (primary guinea pig anti-insulin antibody 1:1000; Dako, Carpinteria, CA) and labeled with secondary antibody (phycoerthrin-conjugated anti-guinea pig IgG; Jackson Immunoresearch, West Grove, PA). Apoptotic cells were identified by fluorescein isothiocyanate–dUTP (Roche) using the TdT enzyme (Deadend Fluorometric TUNEL System; Promega, Madison, WI). The counter-stain, 4'-6-diamidino-2-phenylindole (DAPI; InnoGenex, San Ramon, CA, USA) that intercalates natural double stranded DNA, was used for general identification of nucleated cells. Using fluorescent microscopy, the number of TUNEL+ cells were counted and divided by the number of insulin+DAPI+ nuclei per islet to determine the percent apoptotic β-cells per islet cross-section.

3.3.5 Animals

Immunodeficient C57BL/6-RAG$^{-/-}$ mice (B6.129S7-*Rag1*^{tm1mom}/J) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at the University of Alberta, Heritage Medical Research Centre (HMRC). The animal welfare committee at the University of Alberta approved the ethical use of the mice in this study and all mice were housed and cared for in accordance with the guidelines of the Canadian Council on Animal Care.

3.3.6 *Diabetes induction, blood glucose monitoring, and islet transplantation*

A single intraperitoneal injection of streptozotocin (Sigma-Aldrich, Oakville, ON, Canada) at 200mg/kg induced diabetes in C57BL/6-RAG$^{-/-}$ mice. Diabetes was confirmed by two consecutive random blood glucose level >20mM. Approximately 2000 IEQ were transplanted in the kidney subcapsule and successful engraftment was defined as blood glucose levels <8mM. A return to hyperglycemia was defined as two consecutive random blood glucose levels >15mM.

3.3.7 *Oral glucose tolerance testing*

After overnight fast, dextrose 50% solution (Abbott Laboratories Ltd., Montreal, QC, Canada) was administered at 3mg/kg by oral gavage. The blood glucose level is monitored at 5, 15, 30, 60 and 120 minutes after dextrose load. Oral glucose tolerance testing (OGTT) was carried out at 1, 2, 3, 4, and 5 weeks after initiation of treatment in mice with human islet transplants.

3.3.8 *Human C-peptide determination*

Serum samples were collected by tail vein bleed once per week, for 4 consecutive weeks of oral FTY720 (1mg/kg/d) or vehicle treatment, to determine stimulated C-peptide. The University of Alberta, Special Investigations Laboratory analyzed the serum samples for human C-peptide by ELISA (Roche Diagnostics, Indianapolis, IN, USA). The assay had a lower detection limit of 20pmol/L.

3.3.9 Statistical analysis

Prism version 4.0a (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical calculations. Results are expressed as means ± standard error (SE). Student's t-test was used to analyze two independent groups and a one- or two-way analysis of variance (ANOVA) was used to analyze multiple groups. A p-value <0.05 was considered significant.

3.4 RESULTS

3.4.1 FTY720 does not impair glucose-stimulated insulin secretion in cultured human islets

To assess potential effects of FTY720 on human islet function we began by testing islet insulin secretion in culture (FIGURE 3-1). Human islet glucose-stimulated insulin was not impaired in culture following 48-hour exposure to FTY720 (FIGURE 3-1A). There was no statistically significant difference between the insulin content in any of the groups when cultured in low-glucose or high-glucose medium with or without FTY720 (p=0.40).

To further investigate the impact of FTY720 treatment on human islets, the stimulation index (SI), a relative measure of insulin production in response to glucose stimulation, was calculated by dividing the insulin concentration released in high glucose conditions by the insulin concentration stimulated in low glucose media (FIGURE 3-1B). The SI of human islets in culture with 'physiological' or 'high' FTY720 concentration was not significantly impaired compared to islets cultured in 'control' or 'vehicle' conditions (p=0.82).

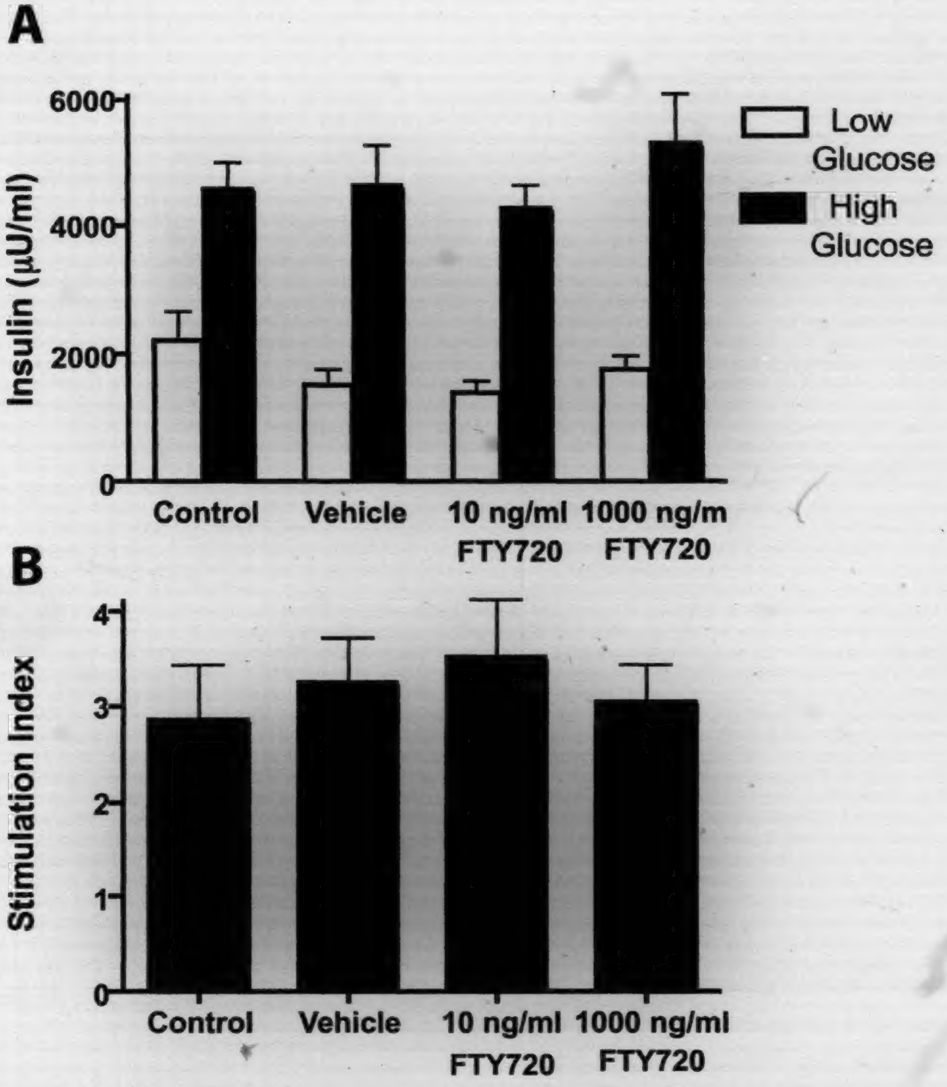

Figure 3-1 FTY720 does not affect glucose-stimulated human islet insulin secretion after a 48-hour incubation.

Duplicate samples of approximately 500 human IEQs were cultured in medium alone 'control', vehicle, or physiological FTY720 (10ng/ml) or high FTY720 (1000ng/ml) at 22°C, 5% CO_2 for 48 hours before incubation with low- or high-glucose at 37°C, 5% CO_2 over a 2 hour period for insulin static assays. **A.** Glucose-stimulated insulin secretion of cultured islets is not impaired by the presence of either physiological (10ng/ml) or high concentration (1000 ng/ml) FTY720 compared to control or vehicle-treated islets, and there was no statistically significant difference between the insulin content of any of the treatment groups when stimulated by low- or high-glucose medium (p=0.40). **B.** The stimulation index (SI), a measure of the relative insulin secretion in response to glucose stimulation, for each group is represented. Although islets incubated with the physiological concentration of FTY720 had the highest SI suggesting improved function, this difference was not statistically significant from control- or vehicle-treated groups (p=0.82). Results represent six individual islet preparations and eight separate insulin static assays.

3.4.2 The presence of FTY720 in culture does not increase the frequency of apoptosis in human islets

While FTY720 did not affect glucose stimulated insulin secretion, it remained possible that FTY720 may have detrimental effects (e.g. apoptosis induction) on some islets but beneficial effects on others, leading to an overall unchanged insulin response. We tested whether FTY720 may induce apoptosis in cultured islets. Representative islet cross-sections demonstrated low TUNEL+ apoptosis staining with or without FTY720 *in vitro* (FIGURE 3-2A). In fact, human islets incubated for 48 hours in FTY720 at 10ng/ml had the lowest number of fluorescent green TUNEL+ cells per islet cross-section, which was significantly less than untreated 'control' islets ($p<0.05$), and high dose FTY720 treated islets ($p<0.05$) (FIGURE 3-2B). However, islets treated with FTY720 at the physiological dose did not have significantly lower β-cell apoptosis compared to vehicle-treated islets ($p>0.05$) (FIGURE 3-2B).

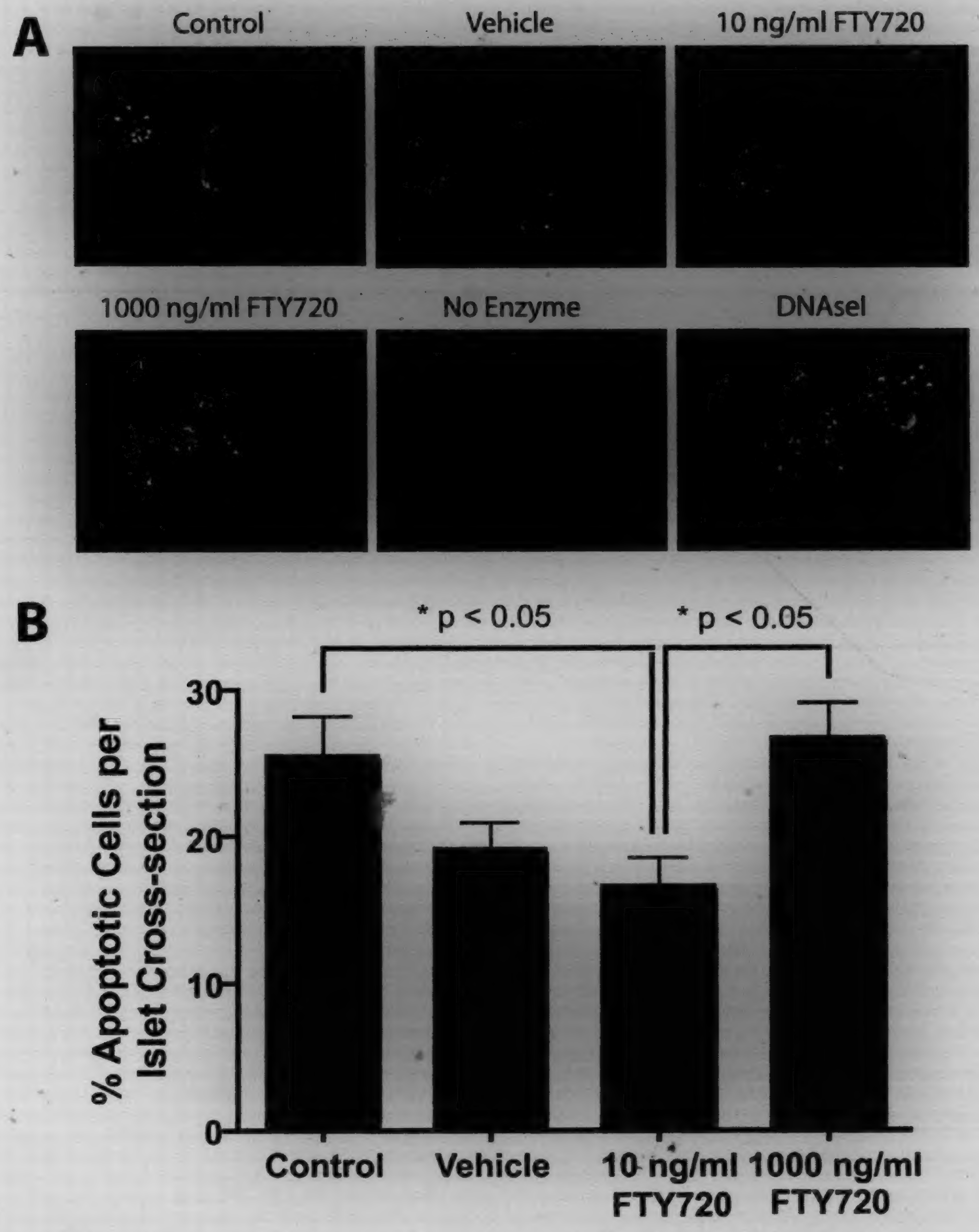

Figure 3-2 FTY720 does not increase the overall number of apoptotic β-cells in cultured islets.

Embedded sections of control-, vehicle-, physiological FTY720-, and high FTY720-treated islets were obtained after 48-hour culture at 22°C, 5% CO_2. **A.** Apoptotic cells are TUNEL+ (fluorescein isothiocyanate) green, insulin+ staining is (phycoerythrin) red, and nuclear staining is (DAPI) blue. Representative islet cross-sections show low numbers of TUNEL+ staining in all treatment groups including control, vehicle, physiological FTY720 (10 ng/ml), and high concentration FTY720 (1000 ng/ml). 'No enzyme' is a negative control where no TdT enzyme was added during TUNEL staining. The 'DNAseI' served as a positive control where DNAseI was intentionally added to introduce nick-ends to the DNA for labeling. **B.** After 48-hour culture, the percent apoptotic β-cells per islet cross-section was lowest in islets incubated with FTY720 at 10ng/ml, which was significantly lower than that in control ($p<0.05$) and high concentration FTY720 at 1000ng/ml ($p<0.05$), but was not statistically different from vehicle-treated islets ($p>0.05$). There is no statistically significant increase in the frequency of apoptosis associated with exposure to FTY720, even at the highest concentration, compared to control or vehicle incubation. Results represent three different islet preparations and three separate TUNEL assays. At least 50 islet cross-sections were examined from each treatment group.

3.4.3 FTY720 does not affect blood glucose levels in immunodeficient mice transplanted with human islets

Although FTY720 did not appear to have any toxic effects on islets *in vitro*, lack of toxicity *in vitro* does not necessarily indicate that islets *in vivo* will also be unaffected. We therefore evaluated the impact of FTY720 administration on human islets transplanted (2000 IEQ) into chemically-induced diabetic immunodeficient mice both immediately after transplantation and delayed at 10-days after engraftment. The time required to achieve normoglycemia or engraftment was not significantly impaired in the presence of vehicle (n=9) or FTY720 (n=8) when compared to control (n=17) ($p=0.38$) (FIGURE 3-3A). Immediate exposure to FTY720 (1mg/kg/d) (n=8) as shown in FIGURE 3-3B, did not impair average glycemic control compared to vehicle-treated mice (n=9) ($p=0.54$) over 50 days. Likewise, mice receiving delayed treatment (FIGURE 3-3D) did not have significant differences in average random blood glucose levels when exposed to vehicle (n=8) or FTY720 (n=9) ($p=0.32$). Individual serum glucose levels (FIGURE 3-3C & 3-3E) are consistent with average levels throughout the 50-day follow-up period, with no evidence of decay in graft function over time. At the time of nephrectomy and islet graft removal (indicated by the arrow heads) mice returned to hyperglycemia confirming the function of the grafts (FIGURE 3-3B, 3-3C, 3-3D, & 3-3E)

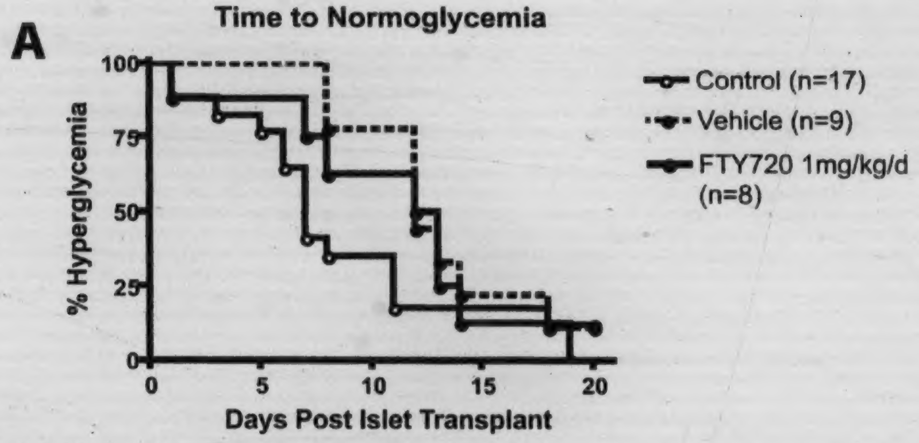

Figure 3-3A

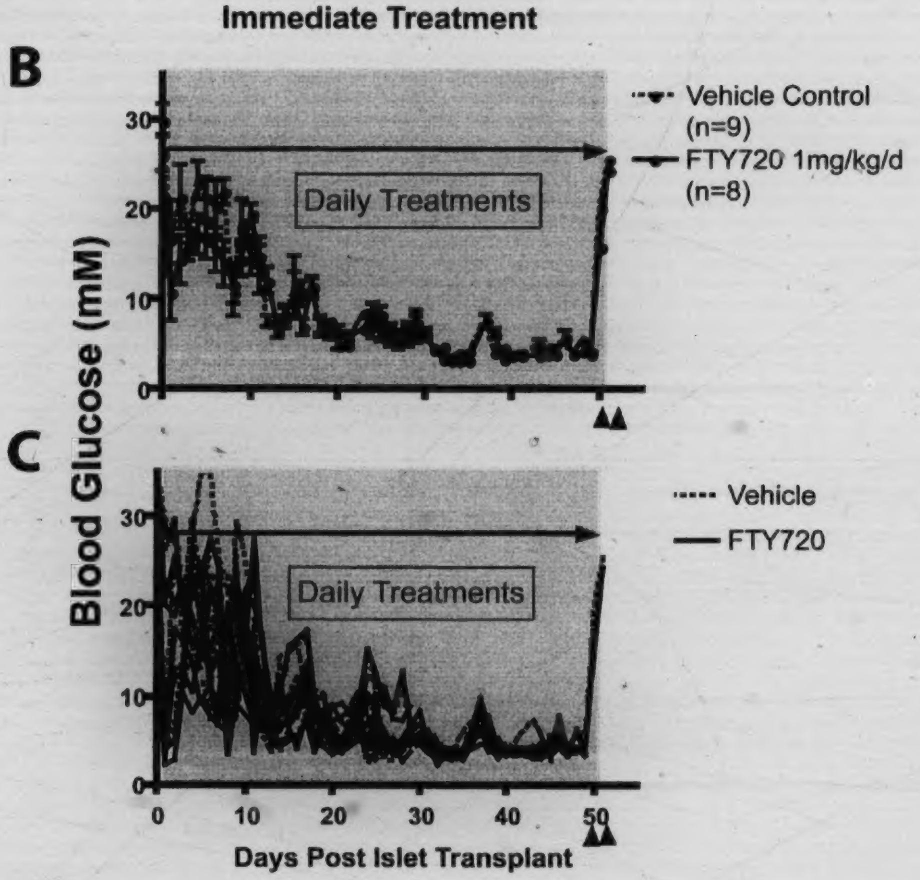

Figure 3-3B & 3-3C

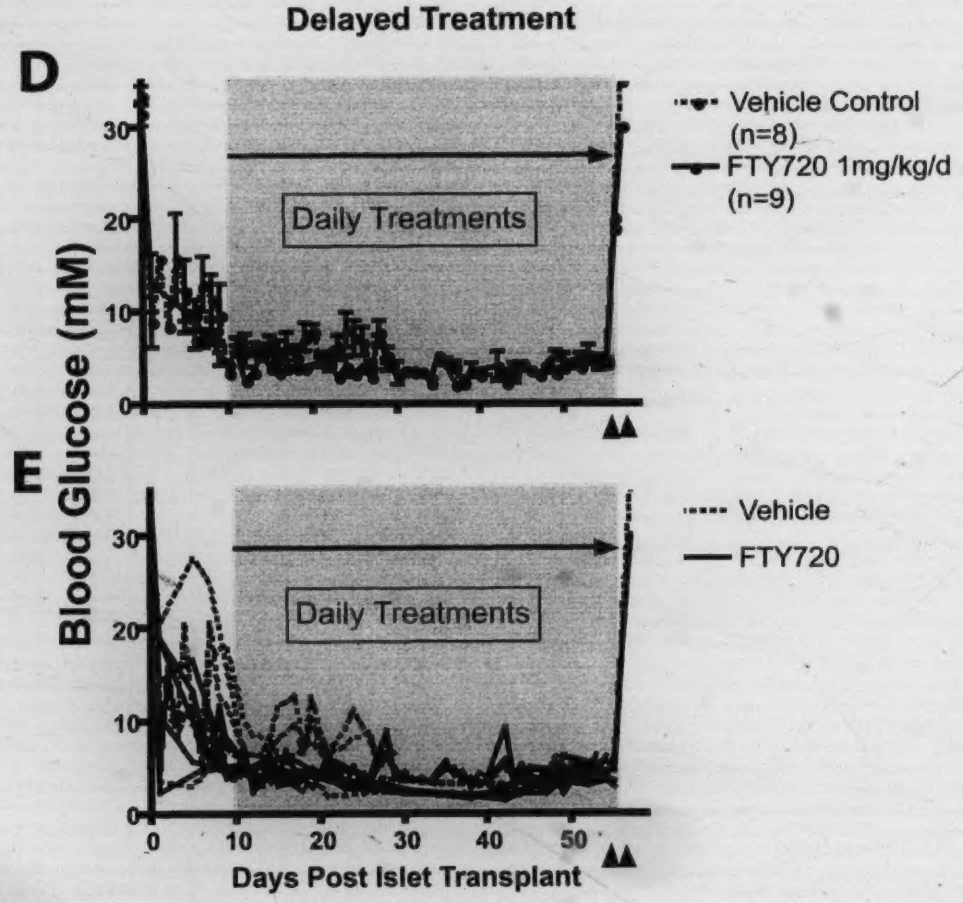

Figure 3-3D & 3-3E

Figure 3-3 FTY720 does not affect non-fasting blood glucose levels.
A. FTY720 does not impair engraftment of human islets compared to control or vehicle treated groups (p=0.38) **B.** In the immediate treatment group, the mean blood glucose levels of mice treated with FTY720 was not impaired compared to vehicle-treated mice (p=0.54). **C.** The individual random blood glucose levels confirm that daily administration of FTY720 does not adversely affect glycemic control over the 50-day follow-up period. **D & E.** Similarly, mice receiving delayed treatment starting at day 10 after transplantation did not show impaired glucose control between vehicle- and FTY720-treated groups over time (p=0.32). After the initiation of daily gavage treatment with either vehicle or FTY720 the random blood glucose levels remained stable with little fluctuations among treatment groups. Arrowheads indicate time of nephrectomy. All animals returned to hyperglycemia after human islet graft removal. Results represent random blood glucose levels in a total of 34 mice transplanted with four different human islet preparations.

3.4.4 *Oral glucose tolerance testing and human C-peptide levels revealed no toxic effects associated with FTY720 administration on the function of human islets in vivo*

To evaluate the function of human islet grafts *in vivo* during exposure to FTY720, transplanted mice were subjected to weekly oral glucose tolerance testing (OGTT), with calculation of the area under the curve (AUC), following 1, 2, 3, 4, and 5 weeks of therapy (FIGURE 3-4A & 3-4B). At 'week 1' of treatment with either 'vehicle' (n=13) or FTY720 (1mg/kg/d) (n=14), the OGTT curves and AUC were not significantly different (p=0.25 and p=0.47, respectively). Analysis of the human islet function at weeks 2, 3, 4, and 5 weeks revealed similar outcomes, with no statistical difference between the FTY720-treated mice and vehicle-treated mice in terms of OGTT curves (p>0.11) or AUC (p>0.46).

While glucose tolerance testing can be used to evaluate graft function over time, it does not reflect changes in insulin reserve and thus relative β-cell mass within an islet graft. In an effort to detect changes in graft β-cell mass over time, the glucose-stimulated human C-peptide levels were measured in vehicle- and FTY720-treated human islet graft recipients after 1, 2, 3, and 4 weeks of treatment. The average stimulated human C-peptide secretion following 1 week of treatment for vehicle-treated mice (n=18) and FTY720-treated mice (n=16) was not significantly different (FIGURE 3-4C; p=0.36). Similar results were observed after 2, 3, and 4 weeks of treatment (FIGURE 4C; p=0.52, p=0.06, p=0.23, respectively). No decline in human C-peptide levels was observed during the 4 week FTY720 therapy period, and was not determined to be significantly different than the concurrent cohort of vehicle-treated mice (FIGURE 4D; p= 0.14).

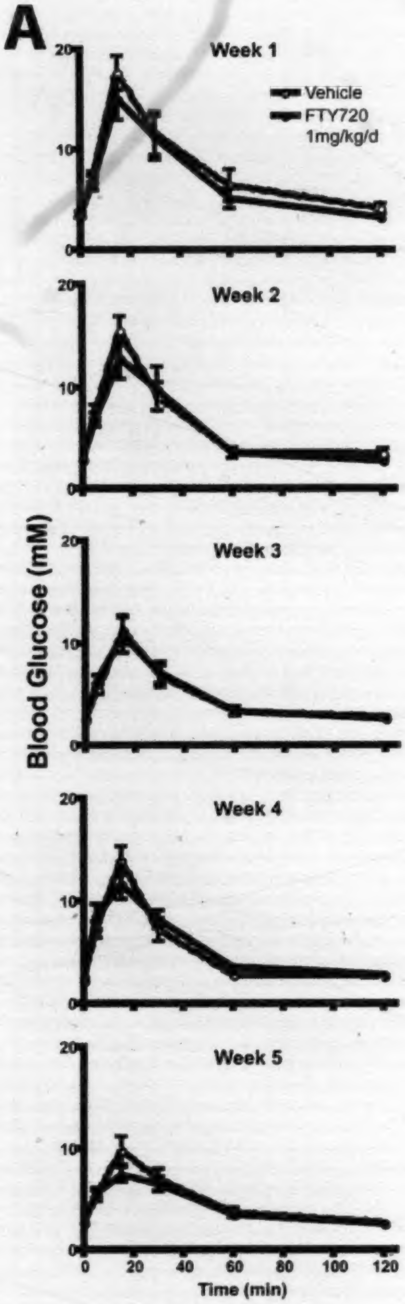
A
Week 1
Vehicle
FTY720
1mg/kg/d
Week 2
Week 3
Week 4
Week 5
Blood Glucose (mM)
0
10
20
0
20
40
60
80
100
120
Time (min)

Figure 3-4A

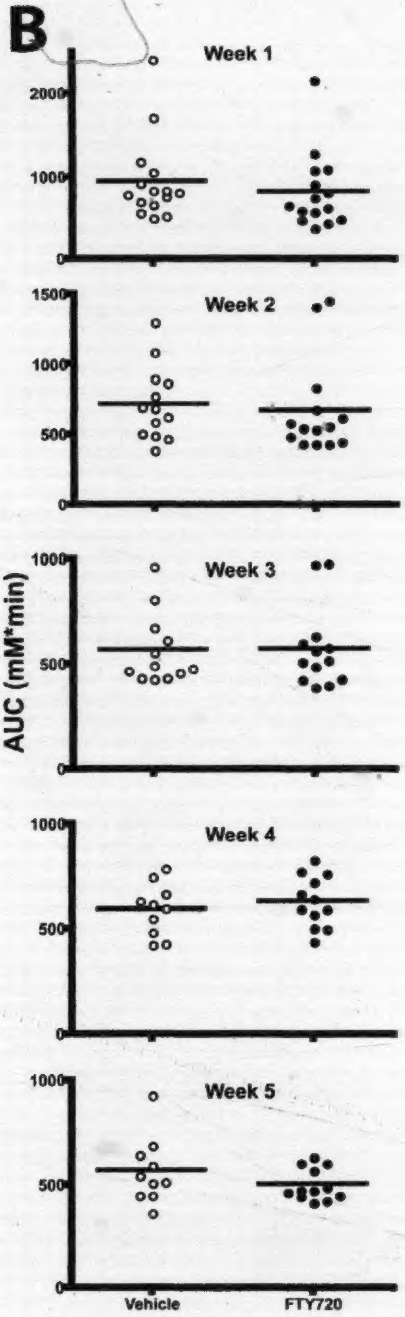
B
Week 1
2000
1000
0
Week 2
1500
1000
500
0
Week 3
1000
500
0
AUC (mM*min)
Week 4
1000
500
0
Week 5
1000
500
0
Vehicle
FTY720

Figure 3-4B

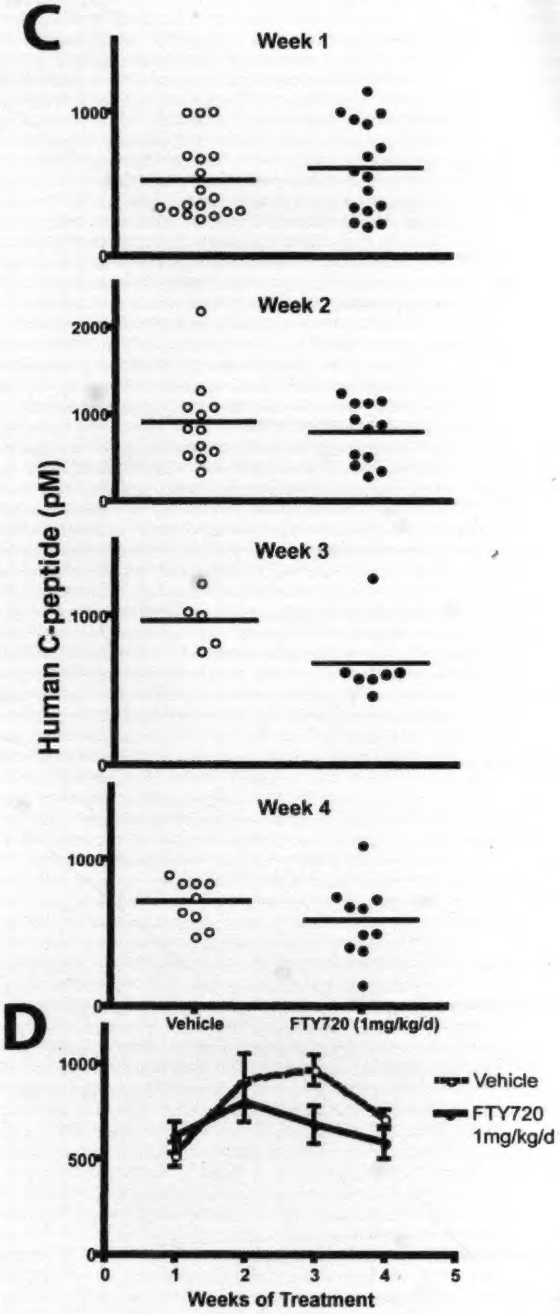

Figure 3-4C & 3-4D

Figure 3-4 FTY720 does not affect Oral Glucose Tolerance Test (OGTT) or Area Under the Curve (AUC) and stimulated human C-peptide levels after initiation of treatment (1 mg/kg/d).

A. The OGTTs demonstrate that the function of human islets *in vivo* after transplantation into immunodeficient mice with chemically induced diabetes was not significantly diminished by exposure to FTY720 when compared to vehicle-treated mice. In fact, mice treated with FTY720 had peak glucose concentrations that were equal to or lower than mice treated with vehicle over the 5-week follow-up, although the differences were not statistically significant. **B.** The AUC, a reflection the functional capacity of islets to regulate glucose levels in the face of a glucose challenge, reinforces that there is no toxic effect of FTY720 administration. There was again no significant change in AUC over the 5-week period of evaluation between mice treated with vehicle or FTY720 at 1mg/kg/d. Results represent OGTT performed in up to a total of 30 mice transplanted with five different human islet preparations. **C.** Daily FTY720 1mg/kg/d or vehicle was administered by oral gavage after euglycemia was confirmed in immmunodeficient mice with chemically induced diabetes. The human C-peptide levels at 1, 2, 3, and 4 weeks after initiation of treatment was not significantly different between vehicle-treated mice compared to FTY720 (1mg/kg/d) treatment (p=0.43). **D.** Although there was a progressive decline in human C-peptide levels in mice treated with FTY720 over the four weeks, this was not statistically different from vehicle-treated mice. Results represent human C-peptide determination in up to a total of 35 mice transplanted with five different human islet preparations.

3.5 DISCUSSION

Immunosuppressive medications including corticosteroids, cyclosporine, and tacrolimus have been linked to islet toxicity and potential diabetogenicity in human patients (14). While FTY720 itself, is unlikely to proceed in clinical transplantation due to side effects including macular edema and bradycardia, it is likely derivatives of FTY720 or emerging S1PR modulators may become useful in the future. Prolonged exposure to FTY720 is required for its clinical benefits and the potentially toxic effects of the drug on human islet function are largely unknown. We therefore have intensively investigated the impact of FTY720 on the function of human islets *in vitro* and *in vivo* following islet transplantation, and have clearly demonstrated an absence of any negative effects.

Human islet function was evaluated after 48 h culture with FTY720. Physiological (10ng/ml) FTY720 was chosen to reflect steady-state (1.0 to 8.8ng/ml) concentrations in human renal transplantation patients (15). A higher concentration of FTY720 was chosen to reflect the conditions possibly experienced by islets delivered into the hepatic portal circulation following oral drug administration (16); however, further experiments could incorporate intraportal grafts to evaluate this *in vivo*. We found no impairment in human islet function after 48 h culture with or without FTY720 exposure in terms of glucose-stimulated insulin or islet viability as determined by apoptosis assay. There was a statistically significant decrease in apoptosis when islets were cultured with the physiological concentration of FTY720 compared to untreated controls, but not compared to vehicle-treated islets, which suggests that the observed anti-apoptotic effect was likely unrelated to FTY720 itself. A 100-fold increase in FTY720 level resulted in a significant increase in β-cell apoptosis compared to the physiological FTY720, but not significantly more than untreated control and vehicle-treated islets. The *in vitro* experiments have several limitations: 1) although, the glucose stimulated insulin assay was carried out at 37°C and *in vivo* studies support the *in vitro* data, islets were exposed to FTY720 at 22°C, which may slow metabolism and delay toxic effects; 2) additional molecular methods, such as measuring caspase levels, may detect finer differences; 3) prolonged culture may be required to show toxic impact of FTY720, but 48 h exposure is sufficient to elicit β cell toxicity in FK506 and cyclosporine (17). Further study could compare FTY720 directly with S1P to examine whether it has the same detrimental effects on GLP-1-stimulated insulin secretion, however, this is beyond the objectives of the current study.

While *in vitro* analysis can provide insight into β-cell function in a controlled tissue culture setting, we chose to challenge our *in vitro* findings by extending these studies into an experimental model of human islet transplantation using chemically induced diabetic immunodeficient mice. It has been previously reported that FTY720 at 1mg/kg/d in rodents corresponds to whole blood trough levels of 20ng/ml (18). To assess engraftment in the presence of FTY720, the time to achieve normoglycemia was evaluated. Although FTY720-treated mice had slightly delayed engraftment compared to control this was not statistically significant (FIGURE 3-3A). In addition (FIGURE 3-3B & 3-3D), there was no difference in blood glucose values in FTY720 treated mice when compared to vehicle-treated mice for the duration of the follow-up period of 50 days. All mice returned to hyperglycemia after nephrectomy and removal of the human islet graft, confirming graft function. Since a progressive decrease in β-cell functional mass over time may not impact blood glucose levels until a significant fraction of the transplanted islet mass has become nonfunctional, more intensive analysis of these mice was carried out using weekly OGTT and stimulated human C-peptide level measurement. There was a trend towards lower AUC (and thus increased β-cell function) was observed in FTY720 treated mice, but this was not statistically significant. This confirms that FTY720 was not toxic to human islets *in vivo* and suggests that with a larger cohort and perhaps with extended follow-up, out to 3 or 6 months, a modest protective effect of FTY720 in human islets might be observed. This hypothesis is supported by results obtained for glucose-stimulated human C-peptide levels in FTY720 treated mice, which were not significantly different than those measured in vehicle-treated mice over time.

While the future of FTY720 in transplantation has been impeded by recent unexpected outcomes in clinical renal transplantation trials, it is likely that FTY720 will be approved for treatment of multiple sclerosis, due to its clear benefit in abrogating disease progression and/or symptoms (19, 20). Development of other S1PR modulators may likely be an important novel immunomodulatory therapy for transplantation. The results in this study suggest that FTY720 or other S1PR modulators will have clear advantages over the diabetogenic and/or nephrotoxic drugs that are currently used in islet transplantation. However, new S1PR modulators with minor structural changes may have significantly different toxicity. Furthermore, different S1PR may exist in islets, possibly modifying the islet susceptibility to toxic effects (10). Therefore, emerging S1PR modulators may require individual examination for human islet toxicity.

Since FTY720 or emerging S1PR modulators may be effective in preventing both autoimmune disease and allograft rejection, this class of drugs may be particularly well suited for the prevention of allograft rejection in islet transplantation for T1DM, which involves a robust allogeneic immune response combined with recurrent graft-specific autoimmunity. Our data suggests that further development of $S1P_1$ receptor specific modulators could result in an extremely useful therapy for islet transplantation.

In summary, no evidence for FTY720-induced β-cell impairment was observed in this study following drug exposure *in vitro* for 48 hours or *in vivo* following daily treatment for 50 days post-transplant.

3.6 ACKNOWLEDGEMENTS

We would like to thank Rena Pawlick for technical assistance with human islet transplantation into the mouse renal subcapsule and Jennifer C. Plester for invaluable assistance with OGTT and blood collection. AMJS is a scholar supported by the Alberta Heritage Foundation for Medical Research (AHFMR), and through a Canadian Institutes of Health Research (CIHR)-Wyeth Clinical Research Chair in Transplantation. CCA is a scholar with the AHFMR. WT is a recipient of fellowships from the AHFMR and the CIHR. Funding for these studies was provided through a Juvenile Diabetes Clinical Center Grant, and through generous donation support from the Roberts Family.

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CHAPTER FOUR

BTLA TARGETING ATTENUATES DISEASE INDUCED BY PD-1 BLOCKADE IN NONOBESE DIABETIC MICE

NOTE: A previous version of this chapter is currently under review in the European Journal of Immunology, and the authors on this paper include: Truong W, Hancock WW, Plester JC, Merani S, Rayner DC, Murphy KM, Anderson CC, and Shapiro AMJ.

4.1 OVERVIEW

The novel coinhibitory receptor B and T lymphocyte attenuator (BTLA) may have a regulatory role in maintaining peripheral tolerance. We hypothesized that antibodies directed against BTLA and programmed cell death-1 (PD-1) coinhibitory pathways would accelerate autoimmune diabetes and may even be synergistic. Non-obese diabetic (NOD) mice at 4 and 10 weeks of age were treated with anti-BTLA mAb, anti-PD-1 mAb, both mAbs, or isotype control and monitored for diabetes development. Anti-PD-1 mAb accelerated the onset of diabetes in NOD mice, while anti-BTLA mAb, unexpectedly, attenuated diabetes induced by anti-PD-1 in a significant proportion of NOD mice. Targeting BTLA with a mAb that acts via a depletional mechanism results in (i) selective reduction of autoaggressive CD4+ T helper cells and B cells; (ii) enhanced proportion of Foxp3+PD-1+CD4+ regulatory T (Treg) cells that may protect against autoimmunity; and (iii) increased cytokines that have a role in Treg generation/function. These data indicate a role for BTLA expressing self-reactive T and B lymphocytes in autoimmune diabetes progression. Hence, depleting strategies targeting BTLA or therapies enhancing the BTLA negative co-signal may prove valuable in treating autoimmune disease and potentially preventing allograft rejection in transplantation.

4.2 INTRODUCTION

Autoaggressive lymphocytes induce development of autoimmune diseases including type 1 diabetes mellitus (T1DM) (1). Full lymphocyte activation requires signals through antigen receptors, either T cell receptor (TCR) or B cell receptor (BCR), and secondary signals through co-signaling molecules (2-6). The balance between positive and negative co-signals regulates the quality, extent, and duration of the immune response. Coinhibitory receptors attenuate, limit, or terminate T cell responses. Three coinhibitory molecules in the immunoglobulin superfamily are identified: cytotoxic T lymphocyte antigen-4 (CTLA-4/CD152) (7), programmed cell death-1 (PD-1/CD279) (8), and B and T lymphocyte attenuator (BTLA/CD272) (9). Although these coinhibitory receptors have structural similarities, their pattern of expression on lymphocytes differs (10-12), likely reflecting their distinct roles during different phases of the immune response.

BTLA expression is low on naïve T cells, is transiently upregulated on activated T cells, but persists on differentiated T_H1 cells (9). Therefore, the function of BTLA is not restricted to primary T cell activation (13), but may also involve ongoing late phases of the immune response, either at the site of an immune reaction or by terminating effector activation within the lymph nodes (9, 11, 12).

BTLA has been implicated in the regulation of lymphocyte activation and peripheral self-tolerance (9, 12-17). *In vitro* BTLA signaling decreases production of IL-2, and attenuates T cell proliferation after TCR engagement (9, 12, 14, 15). Although BTLA-deficient mice have normal development of T and B cells, these mice have increased severity of experimental autoimmune encephalomyelitis (EAE) and increased airway hypersensitivity (9; 16). A recent report demonstrated a significant association between the human BTLA gene with rheumatoid arthritis susceptibility (17). We investigated the role of the BTLA coinhibitory pathway in the development and regulation of autoimmune diabetes in non-obese diabetic (NOD) mice. T1DM involves the interaction of different subsets of lymphocytes and antigen-presenting cells (APC), including CD4+ and CD8+ T lymphocytes, B cells, macrophages and dendritic cells, which together play important roles in the autoimmune response.

In the context of peripheral self-tolerance, loss of coinhibitory signaling is generally associated with autoimmune disease (18-20). In the BDC2.5 TCR transgenic NOD mouse model, anti-CTLA-4 monoclonal antibody (mAb) administered before the development of peri-insulitis accelerated diabetes development (21). PD-1 blockade rapidly precipitated diabetes in NOD mice regardless of age (22). We report that targeting the BTLA coinhibitory pathway using an antibody that depletes BTLA+ lymphocytes protected against the onset of disease in NOD mice and attenuated disease induced by PD-1 blockade.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Female NOD/LtJ and female NOD.129S7(B6)-*Rag1*^{tm1Mom} (NOD-RAG-KO) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and were bred at the University of Alberta. C57BL/6-BTLA$^{-/-}$ (B6-BTLA-KO) mice (9) were originally generated by Dr. Kenneth Murphy and Dr. Theresa Murphy (Washington University, St. Louis, USA), and bred at the University of Alberta. All mice were housed and cared for in accordance with the guidelines of the Canadian Council on Animal Care.

4.3.2 Reagents and treatment protocols

The anti-mouse BTLA mAb (6F7, isotype control mouse IgG1) (23) and the anti-mouse PD-1 mAb (J43, isotype control hamster IgG2) (11) have been described. Pre-diabetic NOD mice at 4 weeks and 10 weeks of age were treated with either anti-BTLA alone, anti-PD-1 alone, combination anti-BTLA and anti-PD-1, or with appropriate isotype control antibodies (n=30 in each group). To determine the *in vivo* dose of anti-BTLA mAb that would block the majority of BTLA receptors, five female NOD mice were treated with a single IP injection, of either 500µg, 250µg, 100µg, and 50µg anti-BTLA mAb (6F7), or no treatment control. Peripheral blood 3 hours after the injection was taken from each of the five NOD mice for flow cytometry analysis with anti-BTLA-PE (6F7) (eBioscience). A dose of 250 µg was sufficient to block the majority of BTLA sites on peripheral B and T lymphocytes up to 7 days (NOT DEPICTED). All antibodies were administered by intra-peritoneal injection (0.50 mg on day 0, and 0.25 mg on days 2, 4, 6, 8, and 10). All purchased monoclonal antibodies and isotype controls were manufactured and purified by BioExpress Inc. (West Lebanon, New Hampshire).

4.3.3 Monitoring diabetes development

Diabetes was defined as random blood glucose levels >15mM for two consecutive days using One-Touch Ultra Glucometer (Johnson & Johnson, New Brunswick, NJ). Female 4- and 10-week-old NOD mice were monitored daily for 3 weeks, then three times weekly until development of diabetes or end of the study. Results represent 30 mice per treatment group.

4.3.4 Histopathology

Pancreases from 4- and 10-week-old female NOD mice were harvested after 10-day course of intraperitoneal injections with anti-BTLA, anti-PD-1, or both anti-BTLA and anti-PD-1. Specimens were stored in formalin and then paraffin embedded. The pancreatic sections were stained with hematoxylin and eosin. Insulitis score was determined by Dr. David Rayner (Faculty of Medicine and Dentistry, University of Alberta), a pathologist blinded to the treatment, and quantification was performed as described previously (24). Briefly, pancreatic islets at 4 and 10 weeks of age were scored according to the following grading system: 0, normal islets; 1, mononuclear infiltration in less than 25% of the islet; 2, 25 to 50% of islet; 3, over 50% of islet; and 4, small, retracted islet with few mononuclear cells. Results represent n=3 from each treatment and age group with at least 10 islets examined.

4.3.5 Adoptive transfer protocol

NOD splenocytes from 6 to 8 weeks of age were prepared, enumerated on a hemocytometer, and flow cytometry was used to determine the % TCR+ population. Approximately 30 million T cells were adoptively transferred via tail vein injection to NOD-RAG-KO mice and treated with three intraperitoneal doses of anti-BTLA (6F7) at 500μg on day 0 and 250μg on days 2 and 4. After treatment, the spleen was harvested and the absolute cell number was determined. Results are representative of three separate experiments with n=5 in each experimental group.

4.3.6 In vitro anti-BTLA binding experiments

NOD splenocytes were pretreated *in vitro* with either IgG1 or anti-BTLA (6F7) mAb, washed, and then stained with either fluorescently-labeled anti-BTLA (6F7), or labeled BTLA-specific mAb (8F4). C57BL/6-BTLA$^{-/-}$ splenocytes served as a negative control.

4.3.7 Flow cytometry analysis

Splenocyte suspensions were obtained for four-color flow cytometry analysis. 10^6 cells were stained with anti-BTLA-PE (6F7), anti-BTLA-biotin (8F4), anti-PD-1 (RMP1-30), anti-PD-1-PE (J43), anti-TCR-FITC, anti-CD4-TC, anti-CD8-APC, anti-B220-APC, and anti-Foxp3-PE. All staining antibodies were purchased from eBioscience and staining performed according to manufactures recommendations.

4.3.8 Immunohistochemistry

After a 10-day course of *in vivo* mAb treatment, pancreases from 4- and 10-week-old NOD mice were harvested, snap-frozen and cryostat sections were labeled using mAbs to BTLA, PD-1, Foxp3, CD4, CD8 and CD19; we purchased mAbs and isotype controls (eBioscience) and an Envision kit (Dako).

4.3.9 Serum cytokine concentration determination

BioSource's Multiplex Bead Immunoassays are solid phase sandwich immunoassay. The assay is analyzed with a Luminex 100™ instrument, which monitors the spectral properties of the capture beads (containing cytokine specific capture antibody) while simultaneously measuring the quantity of associated fluorophore (a biotinylated cytokine specific antibody, incubated with streptavidin-RPE). Assays were performed according to manufacture's protocol and cytokine levels were determined from a standard curve generated using defined cytokine concentrations. Results represent the serum concentrations of the mean of at least 3 mice per treatment group after a 10-day regimen of either anti-BTLA mAb alone, anti-PD-1 mAb alone, or combined treatment.

4.3.10 Statistical Analysis

We used Prism version 4.0a (GraphPad Software Inc., San Diego, CA, USA) for statistical analysis. Survival curve analysis was performed using the log-rank test. Results are expressed as means ± standard error (SE). Student's t-test was used to analyze two independent groups and a one- or two-way analysis of variance (ANOVA) was used to analyze multiple groups. A p-value <0.05 was considered significant.

4.4 RESULTS

4.4.1 Targeting BTLA delays the onset of anti-PD-1 mAb-precipitated disease in NOD mice

To investigate the roles of BTLA and PD-1 in the development of autoimmune diabetes, we administered mAb against PD-1, BTLA, or both to pre-diabetic female NOD mice at 4 or 10 weeks of age. In 4-week-old female NOD mice, anti-BTLA mAb delayed the onset of disease, but this effect was not statistically significant compared to controls (FIGURE 4-1). Consistent with a previous report anti-PD-1 mAb caused disease (22), and increased the rate and incidence of diabetes development in 4-week-old NOD mice compared to controls (p<0.01). Co-administration of anti-BTLA and anti-PD-1 mAbs significantly prevented the accelerated diabetes development caused by anti-PD-1 mAb alone (p=0.017).

The effects of anti-PD-1 mAb were more pronounced in 10-week-old NOD mice than in 4-week-old mice. Anti-PD-1 mAb dramatically precipitated diabetes in 87% of 10-week-old NOD mice, with the majority developing disease before the end of treatment (FIGURE 4-1). Surprisingly, anti-BTLA mAb rescued a significant proportion of 10-week-old NOD mice from the disease induced by anti-PD-1 mAb, reducing the diabetes incidence to 67%, which was significantly different from anti-PD-1 mAb treatment alone (p=0.013).

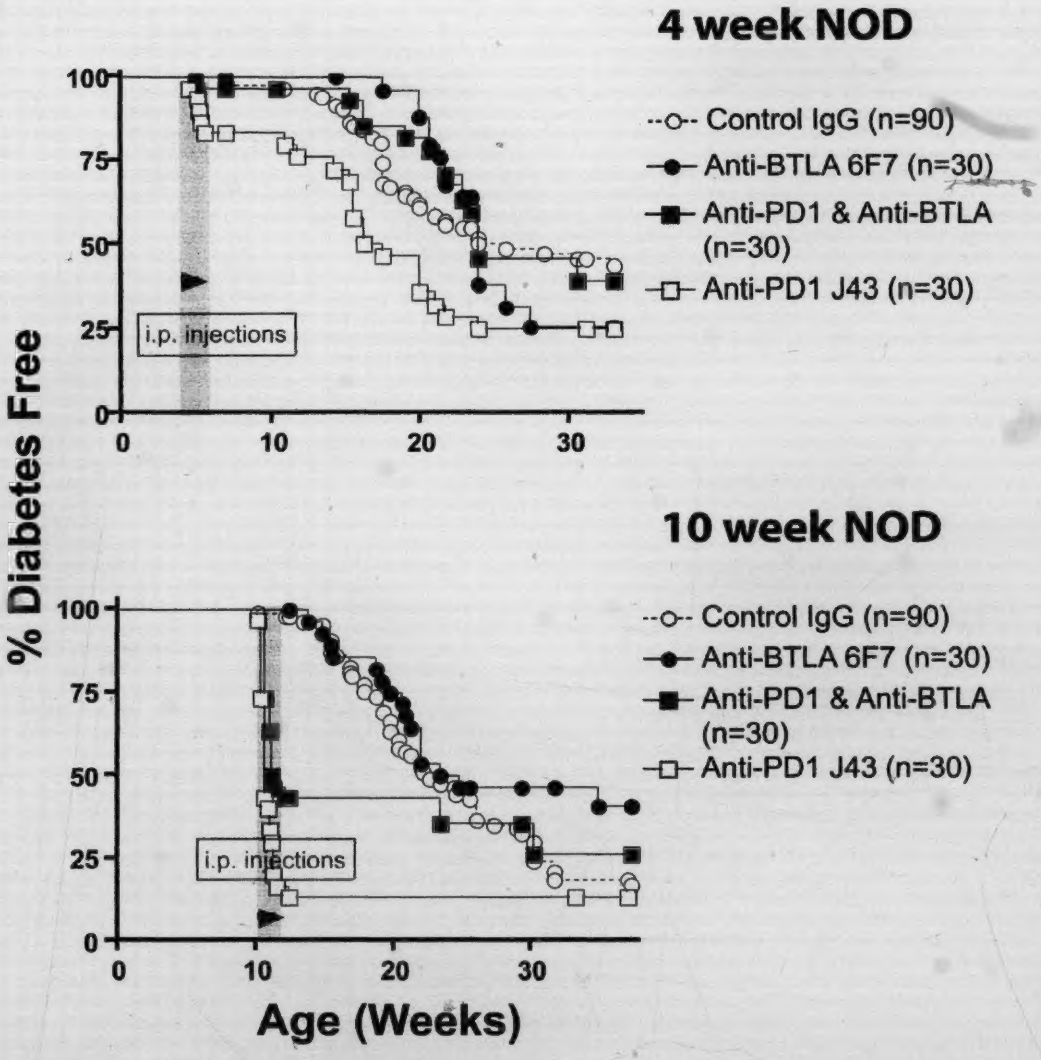

Figure 4-1 BTLA targeting delays the onset of anti-PD-1 mAb-induced diabetes in NOD mice

Diabetes-free survival in 4- and 10-week-old female NOD mice after monotherapy with either anti-BTLA (6F7), anti-PD-1 (J43), or combined therapy (experimental treatment groups are represented by black circles; with n=30 in each of the three groups) compared to isotype control antibody treatment (IgG1, hamster IgG2, or IgG1 and IgG2, respectively, which are represented by white circles; with n=90 in each age group). All p-values are comparisons with respective isotype controls.

4.4.2 Anti-BTLA mAb protects against insulitis induced by anti-PD-1 mAb in pre-diabetic NOD mice

To assess whether the protective effects of anti-BTLA mAb on disease development in NOD mice correlated to changes within the islets, pancreases from 10- or 4-week-old NOD mice treated with mAb to BTLA, PD-1, or both mAbs, were harvested for pathologic comparison to isotype-treated controls. In 10-week-old NOD mice as observed with previous studies (25), invading insulitis was already evident in control islet sections (FIGURE 4-2A). Islets from mice treated with anti-BTLA mAb alone or combined with anti-PD-1 mAb also showed substantial insulitis; however, the insulitis scores were not statistically different from isotype control-treated mice (FIGURE 4-2B). Corresponding to diabetes development, 10-week-old NOD mice treated with anti-PD-1 mAb showed a decline in lymphocyte infiltrates associated with significantly higher insulitis scores compared to controls.

Similar results were seen with 4-week-old NOD mice. Minimal or no peri-insulitis was observed in controls (FIGURE 4-2C). Consistent with diabetes development, islets from NOD mice treated with mAbs against BTLA or both BTLA and PD-1 were free of lymphocyte infiltrates and lacked appreciable peri-insulitis, and insulitis scores confirmed that islet infiltration was not statistically different from that of controls (FIGURE 4-2D). In contrast, NOD mice treated with anti-PD-1 mAb alone exhibited significant insulitis, with insulitis scores statistically higher than controls.

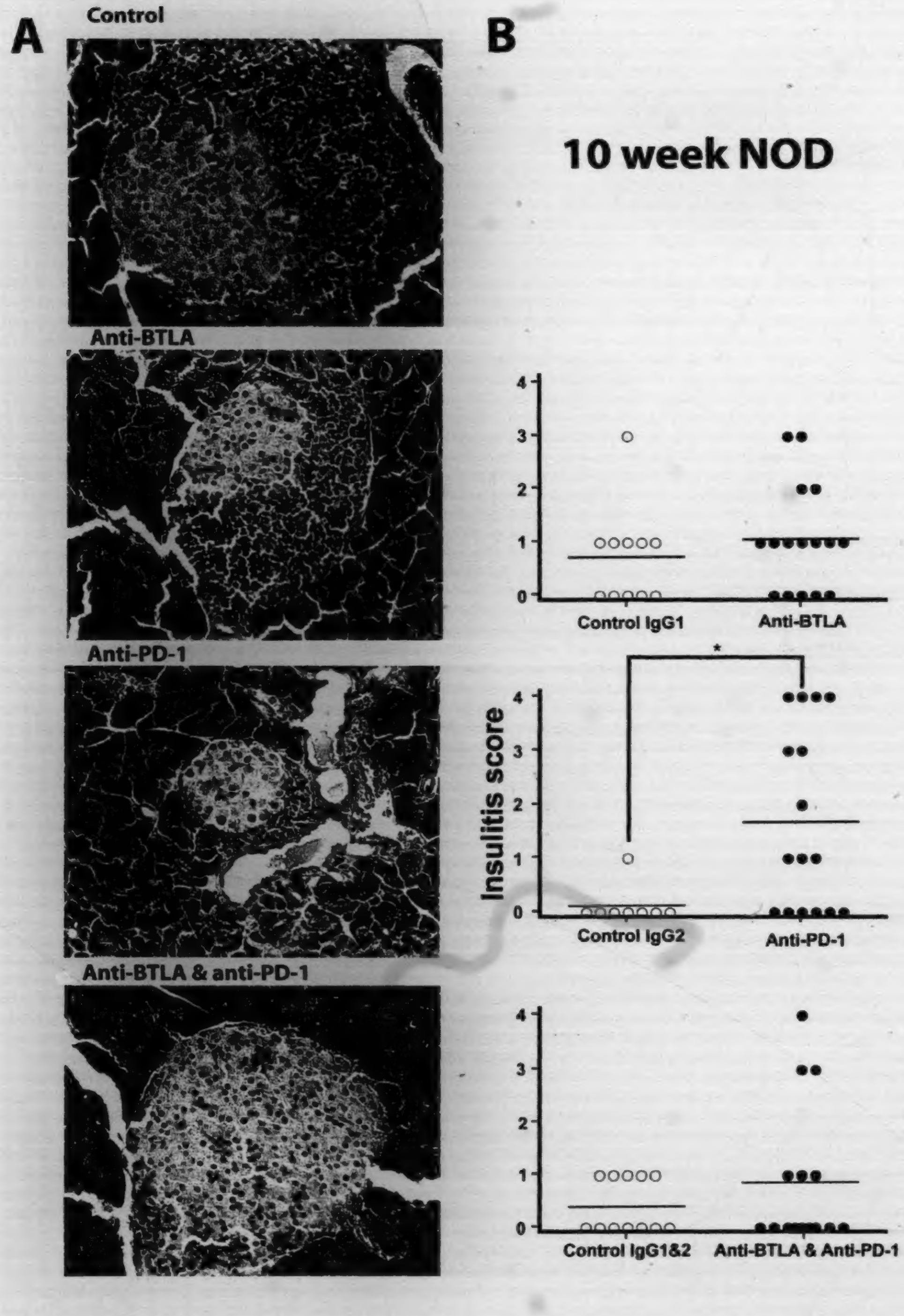
A
Control
Anti-BTLA
Anti-PD-1
Anti-BTLA & anti-PD-1
B
10 week NOD
Insulitis score
0
1
2
3
4
Control IgG1
Anti-BTLA
*
Control IgG2
Anti-PD-1
Control IgG1&2
Anti-BTLA & Anti-PD-1

Figure 4-2 A & B.

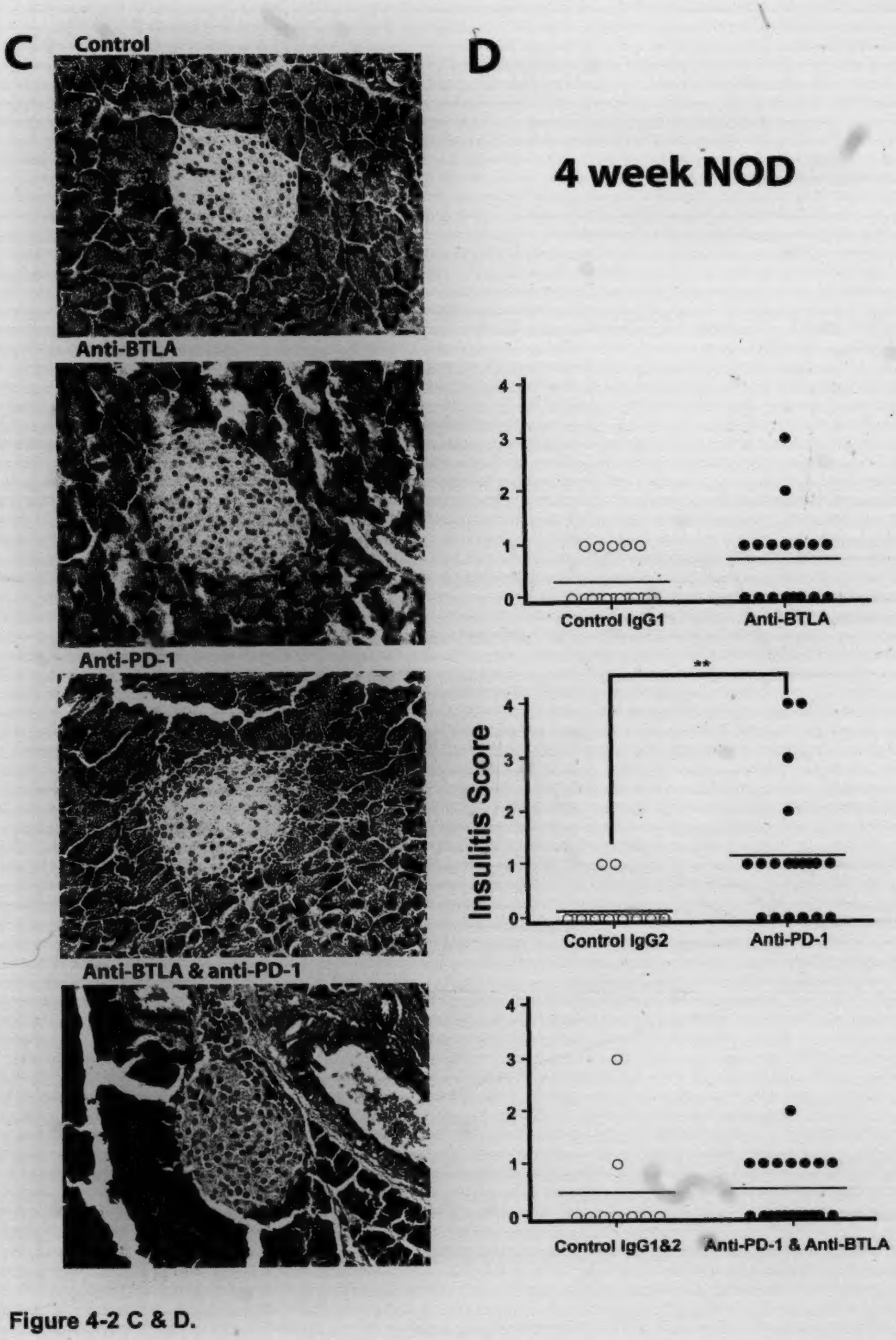
C
Control
Anti-BTLA
Anti-PD-1
Anti-BTLA & anti-PD-1
D
4 week NOD
Insulitis Score
0
1
2
3
4
Control IgG1
Anti-BTLA
**
Control IgG2
Anti-PD-1
Control IgG1&2
Anti-PD-1 & Anti-BTLA

Figure 4-2 C & D.

Figure 4-2 Anti-BTLA mAb protects islets from invading insulitis precipitated by anti-PD-1 mAb

Pancreases were harvested from 10-week-old female NOD mice following 10-day mAb treatment (all p-values represent comparisons with appropriate isotype controls): Black circles represent experimental groups, and white circles represent controls. **(A)** Representative H&E-stained islet sections from control IgG-treated, anti-BTLA, anti-PD-1, or both anti-BTLA and anti-PD-1-treated mice, respectively (all stained with hematoxylin and eosin; x400). **(B)** Insulitis scores after anti-BTLA or IgG1 isotype control administration were not significantly different (p=0.38). Anti-PD-1-treated mice had insulitis scores that were significantly higher than IgG2 isotype controls (*p=0.019). In 4-week-old female NOD mice: **(C)** representative H&E-stained islet sections from anti-BTLA, anti-PD-1, or both anti-BTLA and anti-PD-1-treated, and IgG-treated mice, respectively. **(D)** Insulitis scores after injection with anti-BTLA or isotype control IgG1 were not statistically different (p=0.18). The insulitis scores in anti-PD-1-treated mice were significantly higher than IgG2 isotype controls (**p=0.0011). There was no significant difference in insulitis scores after combination treatment with anti-BTLA and anti-PD-1, or isotype control IgG1 and IgG2 (p=0.44).

4.4.3 Anti-BTLA mAb reduces BTLA expressing B and T lymphocytes in vivo

To explore the mechanism of anti-BTLA mAb (6F7) action on lymphocytes, we assessed expression of BTLA receptors on B and T cells using a second anti-BTLA mAb (8F4) (23). To confirm that the binding of 8F4 was not affected by saturating amounts of anti-BTLA mAb (6F7) (FIGURE 4-3A & 4-3B), splenocytes were pretreated *in vitro* with either anti-BTLA (6F7) or isotype control (IgG1) prior to staining with anti-BTLA (6F7) or anti-BTLA (8F4). C57BL/6-BTLA-/- splenocytes were used to confirm specificity for BTLA (FIGURE 4-3A & 4-3B). Flow cytometry analysis showed that the binding of anti-BTLA (8F4) (FIGURE 4-3A) after pretreatment with anti-BTLA (6F7) was equal to binding after pretreatment with control (IgG1). Conversely, FIGURE 4-3B demonstrates that NOD splenocytes exposed to the same concentration of anti-BTLA (6F7) were saturated and anti-BTLA (6F7) can no longer bind to BTLA on the surface of splenocytes compared to pretreatment with control (IgG1). These results indicate that the labeled 8F4 anti-BTLA mAb can be used to monitor changes in BTLA expression *in vivo* regardless of the presence of 6F7 anti-BTLA mAb.

The percent BTLA expression on the surface of B and T lymphocytes in NOD mice was dramatically reduced after a 10-day treatment with anti-BTLA (6F7) (FIGURE 4-3C). The overall BTLA expression in T cells from anti-BTLA mAb-treated mice was significantly lower than T cells from control-treated mice. Less than 10% of CD8+ T cells, contrasted with over 30% of CD4+ T cells, expressed BTLA; and, after anti-BTLA (6F7) mAb treatment the BTLA expression was significantly lower in both CD8+ and CD4+ T cells. The highest expression of BTLA was on B cells and surface expression was significantly lower after anti-BTLA (6F7) mAb treatment.

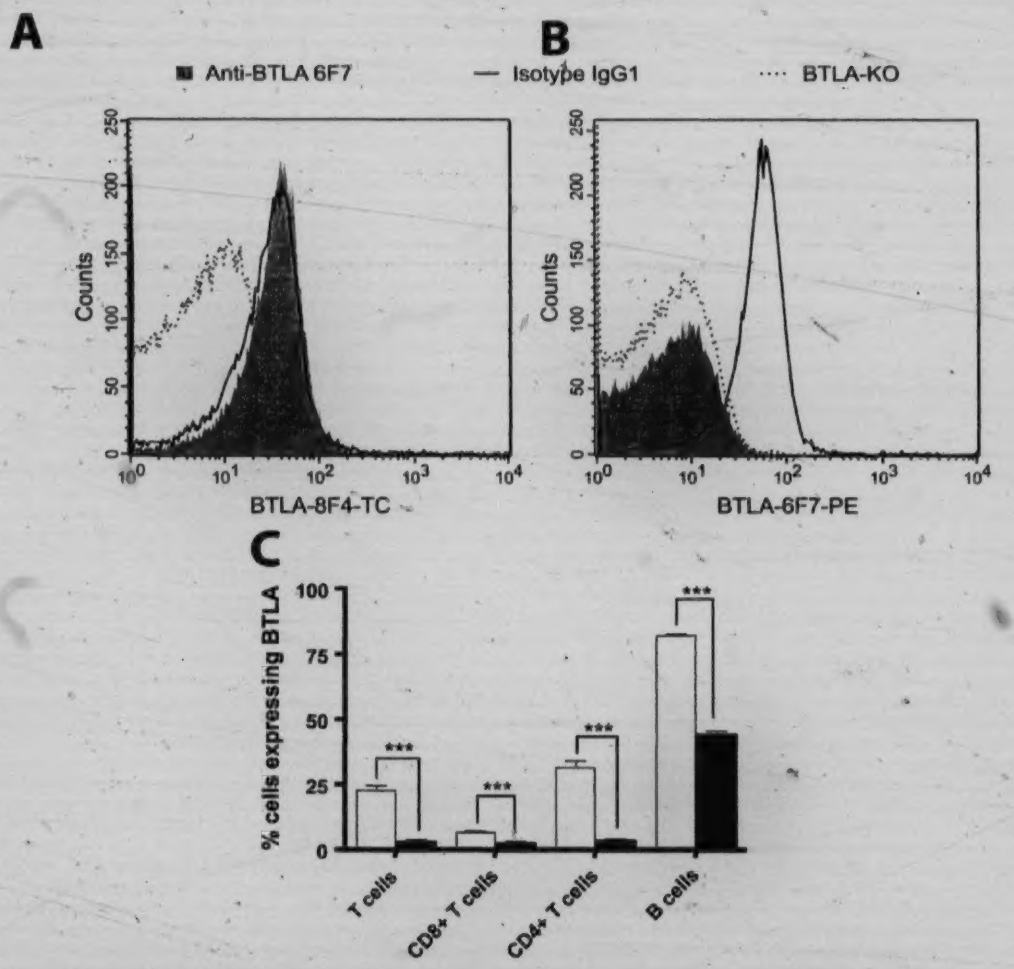

Figure 4-3 Anti-BTLA mAb reduces BTLA expressing B and T lymphocytes
NOD splenocytes were incubated *in vitro* with either IgG1 or anti-BTLA (6F7) mAb, and then stained with anti-BTLA (6F7) or anti-BTLA (8F4). B6-BTLA-KO splenocyte staining is represented by the dotted line. **(A)** Labeled anti-BTLA mAb 8F4 binding to BTLA receptors was unchanged after *in vitro* exposure to anti-BTLA 6F7 (represented by shaded histogram) compared to isotype control IgG1 (represented by the solid black line). **(B)** Conversely, there was no labeled anti-BTLA 6F7 binding after *in vitro* incubation with anti-BTLA mAb 6F7 (represented by shaded histogram), while binding was demonstrated after isotype control IgG1 exposure (represented by the solid black line). **(C)** The percent BTLA expression as determined by anti-BTLA 8F4 staining on lymphocytes after *in vivo* administration (24h after last dose) of isotype control IgG1 (represented by the open bar) was significantly higher than expression after anti-BTLA 6F7 (represented by the closed bar) in NOD mice (***p<0.001). BTLA expression is significantly reduced by anti-BTLA mAb in all lymphocyte subsets. All p-values are comparisons with isotype control.

4.4.4 Anti-BTLA mAb depletes BTLA+ lymphocytes and increases the proportion of Foxp3+PD-1+CD4+ T lymphocytes

The decreased percentage of BTLA surface expression might be due to either: (i) downregulation or internalization of the BTLA receptor; (ii), depletion of the absolute number of cells expressing BTLA; or (iii), sequestration of cells within a lymphocyte compartment. To address these possibilities, we adoptively transferred splenocytes from NOD mice into NOD-RAG-KO recipients, so as to monitor lymphocyte number in the absence of new thymic and bone marrow emigrants. After adoptive transfer, NOD-RAG-KO recipients received isotype control IgG1 or anti-BTLA mAb treatment. The mean splenocyte number was not statistically different between isotype control-treated recipients compared to anti-BTLA mAb-treated mice (FIGURE 4-4A). However, consistent with the percent reductions in BTLA seen in NOD mice (FIGURE 4-3C), the total numbers of T and B lymphocytes expressing BTLA were significantly reduced after anti-BTLA mAb treatment. When comparing absolute lymphocyte counts, the average number of B cells recovered from NOD-RAG-KO recipients treated with anti-BTLA mAb was 73% lower than control (FIGURE 4-4B). Similarly, T lymphocyte numbers from anti-BTLA-treated mice were 29% lower than controls. In anti-BTLA mAb-treated mice, CD4+ T cells were significantly depleted (36% reduction, FIGURE 4-4C), while the CD8+ T cell numbers were lower (10% reduction) but not statistically significant from control. The depletion in T and B cells correlates directly with the percent expression of BTLA by naïve NOD lymphocytes (FIGURE 4-3C).

Certain T lymphocyte subsets were more affected by anti-BTLA mAb treatment. In particular, the percentage of CD4+ T cells expressing Foxp3 or both Foxp3 and PD-1 were increased (FIGURE 4-4D).

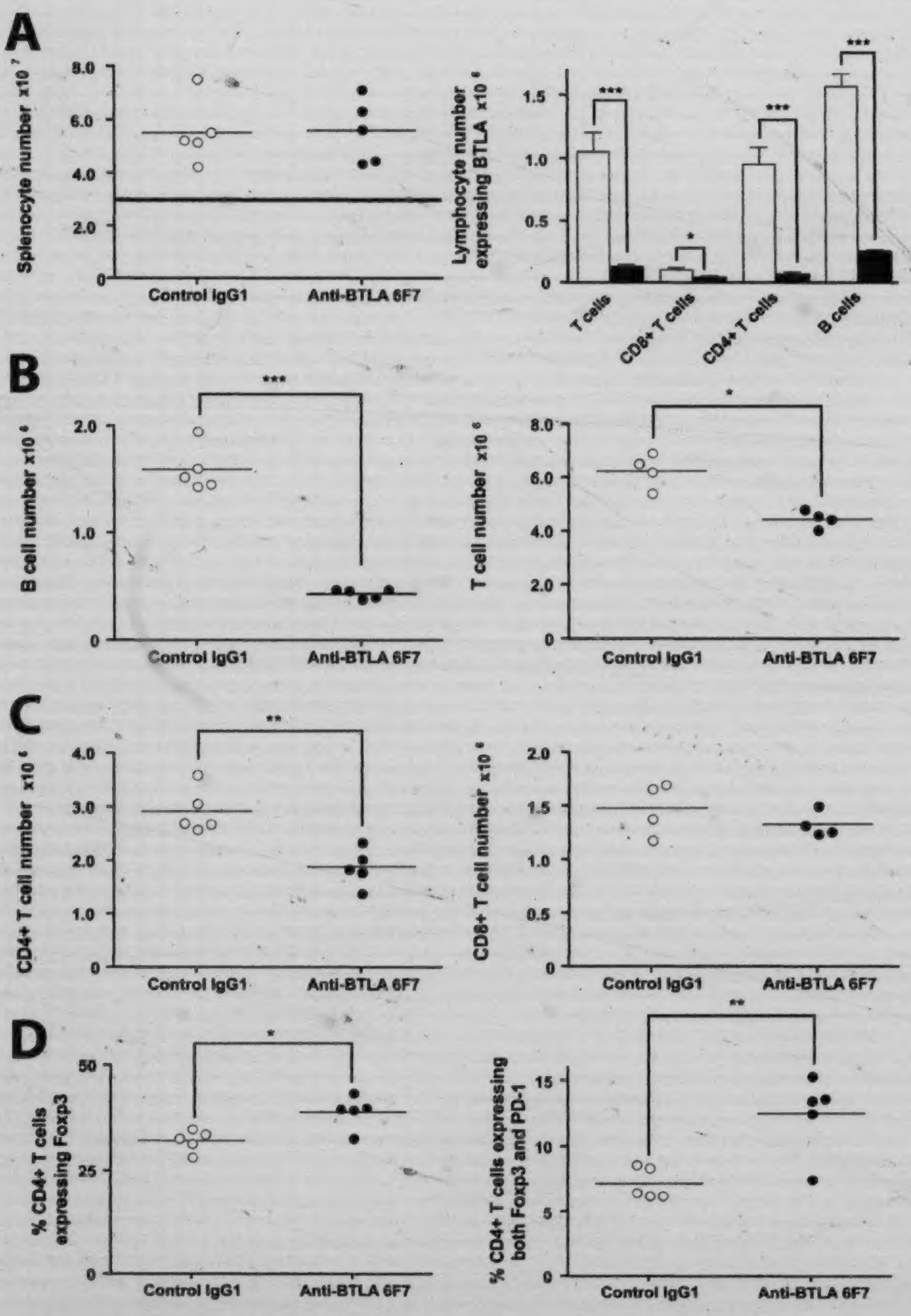

Figure 4-4 Anti-BTLA (6F7) depletes CD4+ T and B lymphocytes and enhances the number of Foxp3+, PD-1+, and CD4+ T cells

(A) The splenocyte number recovered from mice treated with isotype control IgG1 was not significantly different from anti-BTLA-treated mice (p=0.93). BTLA+ lymphocyte numbers including T cells, CD8+ T cells,

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CD4+ T cells, and B cells are significantly reduced after anti-BTLA administration (right panel). **(B)** The absolute B cell number is dramatically decreased in anti-BTLA-treated mice over isotype controls. The total T cell number was significantly lower after anti-BTLA treatment. **(C)** CD4+ T cells were significantly reduced after BTLA modulation. CD8+ T cells were decreased after targeting BTLA receptors but this difference did not reach statistical significance ($p=0.31$). All p-values are comparisons with isotype control ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$). Results are representative of three separate experiments. **(D)** After NOD splenocytes were transferred to NOD-RAG-KO recipients, treatment with anti-BTLA significantly increased the percentage of Foxp3+CD4+ T cells ($^{*}p<0.05$), and similarly, the Foxp3+PD-1+CD4+ T cell percentage is significantly higher with anti-BTLA treatment ($^{**}p<0.01$).

4.4.5 Depleting anti-BTLA mAb decreased islet infiltration by B and CD4+ T cells, while increasing the Foxp3+ and PD1+ lymphocytes

We performed immunohistologic staining of pancreatic samples from 4-week female NOD mice treated with control IgG, anti-BTLA, anti-PD-1, or both anti-BTLA and anti-PD-1-mAbs. Negligible staining was seen for CD4+ T cells, CD19+ B cells or cells expressing BTLA, PD-1 or Foxp3 at this early time-point (FIGURE 4-5). Anti-BTLA mAb therapy led to islet accumulation of small numbers of CD4+, PD-1+, and Foxp3+ cells, consistent with local accumulation of Treg cells, whereas no staining for BTLA or CD19+ B cells was detected. Anti-PD-1 mAb therapy led to significant insulitis with marked islet infiltration by CD4+ T cells and CD19+ B cells, as well as local accumulation of lymphocytes expressing BTLA, PD-1 and Foxp3. Addition of anti-BTLA mAb to the anti-PD-1 mAb resulted in similar immunohistologic findings to that of BTLA mAb alone, with small numbers of CD4+ T cells, no B cells, and expression of PD-1 and Foxp3 but not BTLA by small numbers of mainly peri-islet lymphocytes.

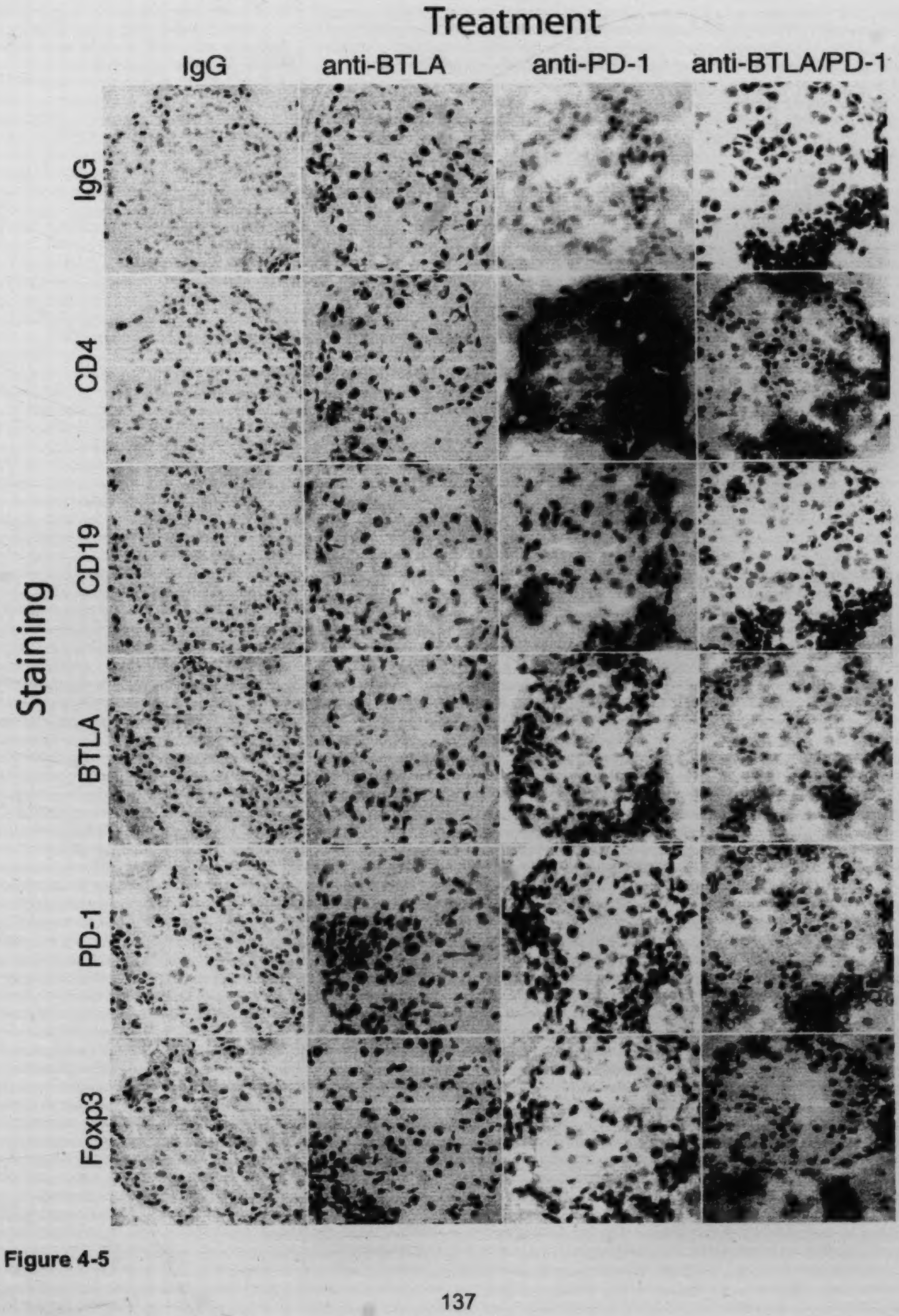
Treatment
IgG
anti-BTLA
anti-PD-1
anti-BTLA/PD-1
Staining
IgG
CD4
CD19
BTLA
PD-1
Foxp3

Figure 4-5

Figure 4-5 Paucity of CD4+ T and B lymphocyte islet infiltrates after anti-BTLA mAb depletion with a relative increase in Foxp3+ and PD-1+ lymphocytes
At 4 weeks, islets from IgG-treated NOD mice lacked infiltrates. Anti-BTLA mAb led to low-level accumulation of CD4+ T cells that lacked BTLA but expressed PD-1 and Foxp3. Anti-PD-1 mAb induced insulitis, with infiltration by CD4+ T cells and CD19 B cells; subsets of infiltrating lymphocytes expressed BTLA, PD-1 and Foxp3. Combined anti-BTLA and anti-PD-1 mAb therapy was associated with a lack of islet B cells or BTLA expression but persistence of a small population of CD4+ PD-1+ Foxp3+ population. Treatment groups are shown in columns and makers of T cells (CD4), B cells (CD19), Tregs (Foxp3) plus BTLA and PD-1 in rows. Each panel is representative of at least 6 islets per section, and 4 animals/treatment group; hematoxylin-stained cryostat sections (x600).

4.4.6 BTLA+ lymphocyte depletion by anti-BTLA mAb alters the T_H1 and T_H2 cytokine profile

Serum cytokine levels were measured by Luminex in NOD mice treated with anti-BTLA, anti-PD-1, or both. The T_H1 cytokine IL-12 was significantly higher in anti-PD-1 mAb-treated NOD mice compared to control treatment or after combined anti-BTLA and anti-PD-1 mAbs (FIGURE 4-6). In contrast, IL-2 and IFNγ concentrations were significantly elevated in mice treated with anti-BTLA mAb or combined anti-BTLA and anti-PD-1 mAbs (FIGURE 4-6). In terms of traditional T_H2 cytokines, the anti-BTLA alone group had increased IL-4 and IL-10 levels relative to control mice.

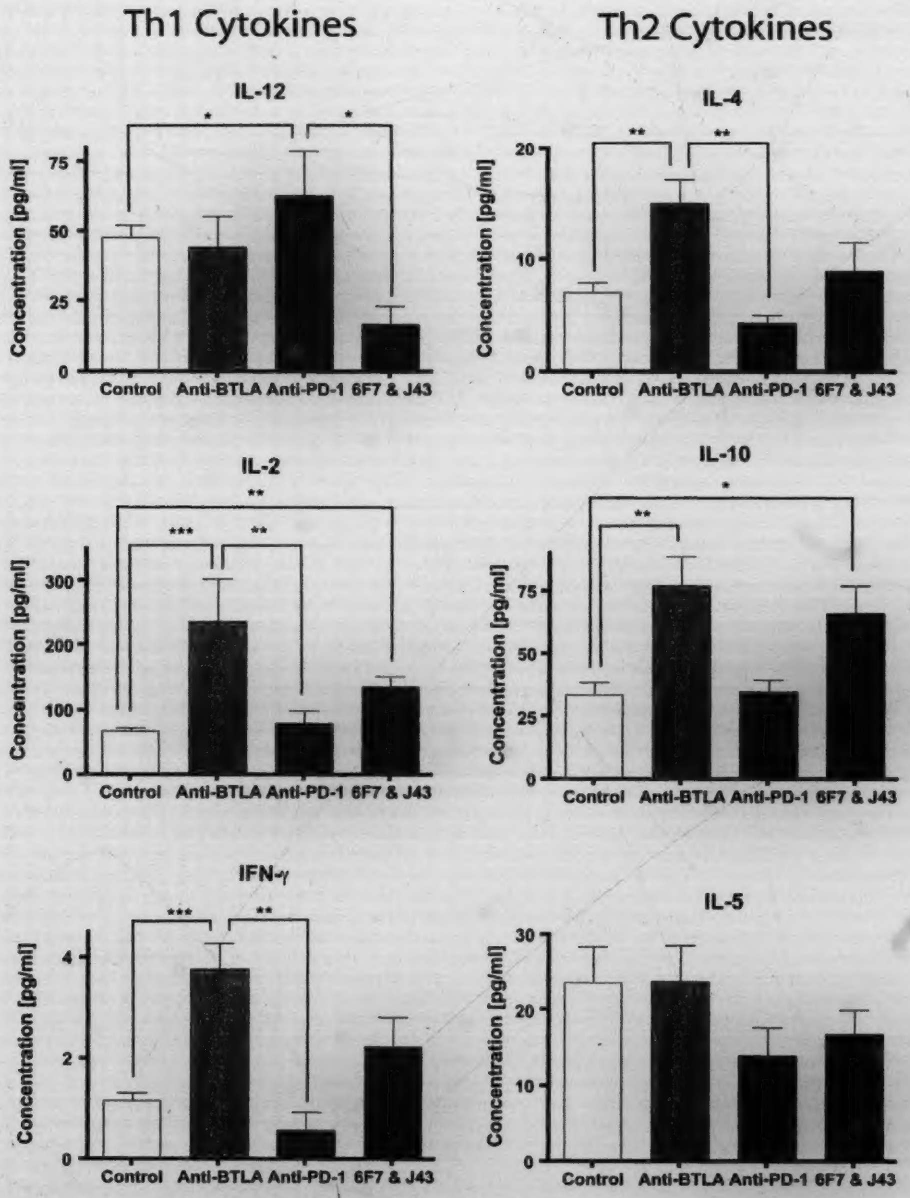

Figure 4-6 BTLA+ depleting anti-BTLA mAb 6F7 and PD-1 blockade with anti-PD-1 mAb J43 significantly alters the balance of serum cytokines in NOD mice

The T_H1 cytokine IL-12 was induced with anti-PD-1 mAb treatment and was significantly higher than control mice and NOD mice treated with both anti-BTLA and anti-PD-1. Traditional T_H1 cytokines IL-2 and IFNγ were higher in anti-BTLA-treated mice compared to control and anti-PD-1-treated NOD mice. The T_H2 cytokines IL-4 and IL-10 were statistically elevated in anti-BTLA-treated NOD mice compared with control, and IL-10 was significantly higher in mice treated with anti-BTLA and anti-PD-1 compared to control. There was no significant difference in the secretion of IL-5. All p-values are comparisons with isotype control (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

4.5 DISCUSSION

BTLA is a negative cosignaling pathway thought important to the control of the late phases of lymphocyte activation and for peripheral self-tolerance (9, 12-17). We report that targeting the BTLA negative cosignaling pathway, unexpectedly, delayed diabetes development and protected against insulitis caused by anti-PD-1 mAb in NOD mice (FIGURE 4-1). The results of anti-BTLA mAb administration in the development of autoimmune diabetes, suggested that the antibody was non-blocking, since blockade of a negative signal would be expected to cause accelerated diabetes development. In fact, anti-BTLA mAb was able to protect islets from anti-PD-1 mAb-induced insulitis (FIGURE 4-2), and anti-BTLA mAb was dominant over PD-1 pathway blockade in autoimmune diabetes.

Anti-BTLA mAb 6F7 did not cross-link *in vitro* to deliver a negative signal through its engagement with BTLA receptor, since proliferation in response to TCR stimulation was not decreased in the presence or absence of anti-BTLA mAb (NOT SHOWN). This argues against agonistic activity of 6F7.

NOD mice expressed BTLA at levels that were consistent with previous reports using C57BL/6 and BALB/c mice (23). Mature B cells constitutively expressed BTLA (83%), and expression was maintained at low levels on mature CD4+ (32%) and CD8+ (7%) T cells in the periphery. BTLA expression was similar between 4- and 10 week-old female NOD mice. Anti-BTLA mAb significantly reduced BTLA expressing peripheral B and T cells.

To determine the mechanism of anti-BTLA 6F7 mAb action, a model of adoptive transfer using splenocytes from female NOD to NOD-RAG-KO recipients revealed that anti-BTLA mAb depleted absolute CD4+ T and B cell numbers (FIGURE 4-4). Moreover, the cell number in each lymphocyte group was depleted by approximately the same percentage that expressed BTLA (FIGURE 4-3). Immunostaining for BTLA+ cells were significantly diminished after treatment with depleting anti-BTLA mAb, corresponding to a significant decrease in CD4+ T and B lymphocyte infiltration of islets, which is evidence against sequestration at the site of inflammation (FIGURE 4-5).

Autoantibodies have been detected in T1DM in both prediabetic and newly diagnosed human patients and murine models (26, 27), implicating B cells in autoimmune diabetes. However, initial studies showed that antibodies do not cause diabetes directly, since administration of autoantibodies isolated from diabetic mice were not able to induce diabetes in recipients (28). Nevertheless, the importance of B cells in autoimmune diabetes is demonstrated by the unexpected finding that NOD mice rendered deficient of B cells were highly resistant to T1DM and reconstitution with NOD B cells reversed this resistance (28-32). B cells may have a significant role as antigen-presenting cells (APCs) (31, 33-35). B cells express the highest levels of BTLA, and are therefore most affected by anti-BTLA mAb 6F7 treatment. Depleting BTLA+ self-reactive B cells may have a significant biological effect of delaying disease in NOD mice.

However, in humans and NOD mice, CD4+ and CD8+ T cells are the major mediators of insulitis causing T1DM (36), and potentially autoreactive T cells could develop to cause disease despite the lack of B cell involvement. CD4+ T cell helper (T_H) cells are critical for cytotoxic T lymphocyte (CTL) development and B cell activation. Following Ag-specific activation of CD4+ T cells, BTLA is rapidly induced, with peak surface expression by day 2 (23). Conceivably, anti-BTLA mAb protection from diabetes development is due to depletion of BTLA+ autoreactive CD4+ T lymphocytes, whereas the effects of anti-PD-1 mAb are due to inhibition of negative signaling via the PD-1 pathway. The unique combination of depleting B and CD4+ T cells makes anti-BTLA mAb 6F7 a novel and potentially useful therapeutic agent not only in the prevention of autoimmune disease, but also in prolonging allograft survival. Alternatively, the development of agonistic mAbs to BTLA might be an effective strategy for suppressing autoreactive T lymphocytes.

Even though absolute numbers of CD4+CD25+ Tregs are diminished in prediabetic NOD mice when compared to mouse strains not susceptible to autoimmune diseases (37). Skewing the Treg to non-Treg ratio may be another mechanism in which anti-BTLA mAb prevents disease progression in prediabetic NOD mice. BTLA-specific mAb enhanced the relative proportion of Foxp3+CD4+ Treg, as well as PD-1+Foxp3+CD4+ T lymphocytes in an NOD mouse, adoptive transfer model (FIGURE 4-5). This, in part, may contribute to its protective impact on NOD mice and in addition, decreasing the effectiveness of anti-PD-1 mAb to precipitate diabetes.

Immunohistologic evaluation of islets from female NOD mice treated with anti-BTLA mAb lacked BTLA staining and confirmed no accumulation of BTLA+ cells at the site of immune reaction within the pancreas. Previous studies have shown that the ligand for BTLA, herpesvirus entry mediator (HVEM), is expressed at high levels in islets (38), and could act to signal through BTLA to prevent autoaggressive T and B cells from causing disease. Notably there was a decrease in CD4+ T and B cells in the pancreas of mice treated with anti-BTLA mAb, which is consistent with the depletion found in adoptive transfer experiments. In addition, there was a modest recruitment of Foxp3+ and PD-1+ cells after anti-BTLA mAb treatment.

Since anti-BTLA mAb selectively depleted CD4+ T and B lymphocytes, we examined whether this change in lymphocyte population alters the cytokine environment compared to control and anti-PD-1-treated NOD mice. Indeed, anti-BTLA mAb was associated with changes in cytokine environment and may partially account for the protection from diabetes induced by anti-PD-1 administration. Unlike cross-linking anti-BTLA mAb used in previous studies *in vitro* (9), the depleting anti-BTLA mAb 6F7 used in our *in vivo* experiments demonstrate a different cytokine profile (FIGURE 4-6). The T_H1

cytokine IL-12 is significantly higher in anti-PD-1-treated NOD mice over control- and combined anti-BTLA- and anti-PD-1 mAb-treated mice. IL-12 an important cytokine in the T_H1 pathway may also play a role in diabetes development, since IL-12 blockade inhibits the development of diabetes in NOD mice and exogenous IL-12 accelerates development (39). Interestingly, serum IFNγ levels are increased in mice treated with anti-BTLA alone or in combination with anti-PD1 mAb. While IFNγ is commonly known as an effector T_H1 cytokine, it also has a negative regulatory role early in T cell responses (40) and is implicated as an important cytokine in Treg function (41, 42), lending support to the hypothesis that anti-BTLA mAb is promoting Treg cell persistence. In addition, anti-BTLA mAb causes an increase in the T_H2 cytokines IL-4 and IL-10 compared to control NOD mice. Exogenous IL-4 injections are capable of preventing T1DM progression in NOD mice (43, 44). Administration of exogenous IL-10 offers disease protection in NOD mice (45). Although treatment with anti-BTLA mAb did not cause a uniform shift in the balance from T_H1 to T_H2 cytokines, the cytokine profiles in anti-BTLA mAb-treated NOD mice showed upregulation of IFNγ and IL-10, cytokines that are favorable to regulatory T cell generation/function and hence prevention of autoimmune diabetes development.

In summary, anti-BTLA mAb 6F7 significantly depleted BTLA+ lymphocytes, and limited the autoimmune destruction of beta cells by self-reactive CD4+ T and B cells in NOD mice. Depleting anti-BTLA mAb delayed the onset of diabetes and skewed the remaining lymphocytes populations towards Tregs, and created an associated cytokine environment that is conducive to Treg function (FIGURE 4-7). In addition, we have demonstrated that anti-BTLA mAb 6F7 is unique, unlike other agents targeting costimulatory molecules, it can affect both autoaggressive CD4+ T and B lymphocytes. The distribution of BTLA expression expands the possible lymphocyte subset pool that could be targeted by BTLA modulation. Therefore, BTLA-specific mAbs may have therapeutic potential not only in autoimmune disease like T1DM but also in tolerance induction in transplantation grafts. Further investigation is required to fully explore the possibility of using such a monoclonal antibody in allotransplantation of islets to treat clinical T1DM, since treatment requires protection from both auto- and alloimmunity.

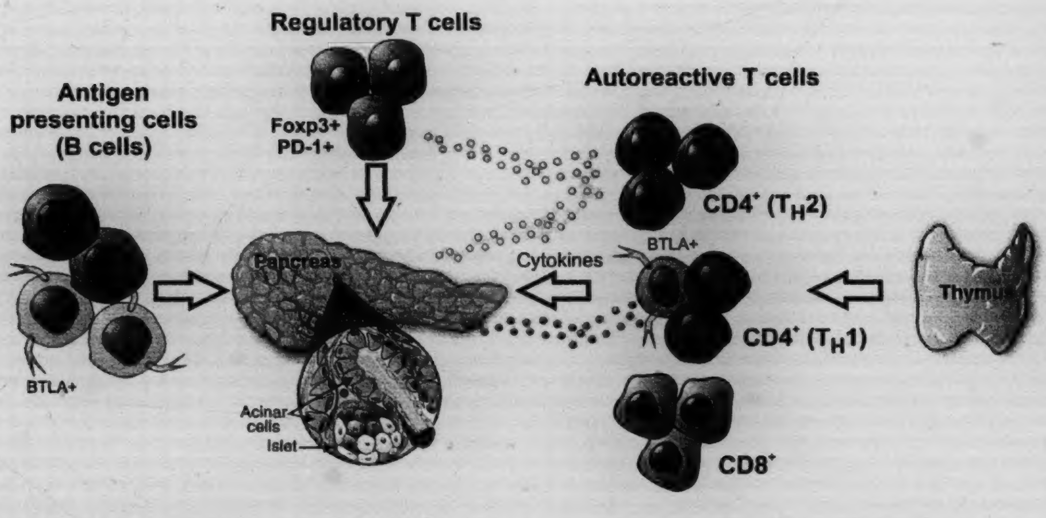
Regulatory T cells
Antigen presenting cells (B cells)
Foxp3+
PD-1+
Autoreactive T cells
CD4⁺ (T_H2)
BTLA+
Cytokines
Pancreas
Thymus
CD4⁺ (T_H1)
BTLA+
Acinar cells
Islet
CD8⁺

Figure 4-7 Mechanisms of action of anti-BTLA mAb 6F7 in the prevention of diabetes development in NOD mice
Treatment with anti-BTLA mAb depletes both BTLA+ CD4+ T and BTLA+ B cells. In addition, it increases the proportion of Foxp3+ PD1+ T cells, and skews the cytokine milieu away from autoimmunity.

4.6 ACKNOWLEDGMENTS

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CHAPTER FIVE

COMBINED COINHIBITORY AND COSTIMULATORY MODULATION WITH ANTI-BTLA AND CTLA4Ig FACILITATES TOLERANCE IN MURINE ISLET ALLOGRAFTS

NOTE: A previous version of this chapter was
accepted for publication and is currently in press in the
American Journal of Transplantation. The authors on this paper include:
Truong W, Plester JC, Hancock WW, Merani S, Murphy TL,
Murphy KM, Kaye JG, Anderson CC, and Shapiro AMJ.

5.1 OVERVIEW

Complex interactions between positive and negative co-signaling receptors ultimately determine the fate of the immune response. The recently identified coinhibitory receptor, B and T lymphocyte attenuator (BTLA), contributes to regulation of autoimmune and potentially alloimmune responses. We investigated the role of BTLA in a fully major histocompatibility complex-mismatched mouse islet transplant model. We report that anti-BTLA mAb (6F7) alone does not accelerate graft rejection. Rather, while CTLA4Ig alone improved allograft survival, the addition of anti-BTLA mAb to CTLA4Ig led to indefinite (>100 d) allograft survival. Immediately after treatment with anti-BTLA mAb and CTLA4Ig, islet allografts showed intact islets and insulin production despite a host cellular response, with local accumulation of Foxp3+ cells. We clearly demonstrate that combined therapy with anti-BTLA mAb and CTLA4Ig mice induced donor-specific tolerance, since mice accepted a second donor-specific islet graft without further treatment and rejected third party grafts. CTLA4Ig and anti-BTLA mAb limited the initial *in vivo* proliferation of CFSE-labeled allogeneic lymphocytes, and anti-BTLA mAb enhanced the proportion of PD-1 expressing T cells while depleting pathogenic BTLA+ lymphocytes. We conclude that targeting the BTLA pathway in conjunction with CTLA4Ig costimulatory blockade may be a useful strategy for promoting immunological tolerance in murine islet allografts.

5.2 INTRODUCTION

Islet transplantation is a viable option for patients with severe forms of type 1 diabetes mellitus (T1DM) (1). With recent reports of declining long-term islet allograft function in T1DM after islet transplantation (2), an immune modulating regimen with minimal β cell toxicity and decreased immunosuppressive side-effects is paramount to preserve islet function and prevent both allograft rejection and recurrent autoimmunity. Traditional glucocorticoid-based therapy has well recognized detrimental impact on islet function *in vitro* and *in vivo* (3, 4). Despite steroid-free protocols using sirolimus (rapamycin), low-dose tacrolimus, and anti-IL2-receptor mAb (daclizumab) induction (1), local islet toxicity and systemic side-effects persist, limiting the application of islet replacement therapy to a small subgroup with severe unstable glycemic control (4, 5). New calcineurin inhibitor-free agents may avoid both diabetogenicity and nephrotoxicity (6, 7).

An attractive strategy to prevent graft rejection is to facilitate tolerance induction by targeting co-signaling pathways. T lymphocytes are instrumental in determining the alloimmune response to transplanted grafts. T cell activation relies on two distinct signals: (i) through an antigen-specific T cell receptor (TCR); and (ii) through co-signaling receptors, which can be either positive or negative. The balance between co-signaling receptors determines the outcome of the immune response, and the TCR signal without costimulation may lead to anergy, apoptosis, or ignorance (8-10). In addition, coinhibitory receptors can limit, or even turn off T cell activation, and may direct the immune response towards tolerance.

The costimulatory receptor CD28 is constitutively expressed on T cells, therefore CD28:B7 costimulatory pathway is important early in T cell activation, and ligation with antigen presenting cells (APC) expressing B7-1 (CD80) and B7-2 (CD86) induces T cell proliferation and effector activation (9). The fusion protein cytotoxic T lymphocyte antigen-4 immunoglobulin (CTLA4Ig) competitively blocks CD28 binding with its ligands and facilitates long-term allograft survival in several transplantation models (11-13).

The emerging importance of negative co-signaling molecules, including CTLA-4/CD152 (14), programmed cell death-1 (PD-1/CD279) (15), and B and T lymphocyte attenuator (BTLA/CD272) (16), adds an additional dimension to the complexity in which T lymphocytes are regulated (17). Programmed cell death ligand-1 immunoglobulin (PD-L1Ig) targeting the PD-1 coinhibitory pathway has been used successfully to enhance anti-CD40L (CD154) induced islet allograft survival (18), and to prolong cardiac allografts in CD28-deficient recipients (19). BTLA is a coinhibitory receptor with structural similarities to CTLA-4 and PD-1 and the negative co-signaling role of BTLA is supported by observations of attenuated lymphocyte responses after *in vitro* cross-linking (20). Although BTLA-deficient mice have normal B and T cell development, these mice have exacerbated responses in the experimental autoimmune encephalomyelitis model (16), and worse disease in a model of allergic airway inflammation (21). BTLA targeting has been implicated in the accelerated rejection of partially-mismatched cardiac grafts but also in prolongation of fully-mismatched allografts (22), and we hypothesize that BTLA negative co-signaling may be important in the context of costimulatory blockade in islet allograft survival.

Herein, we investigate the effects of targeting the BTLA coinhibitory pathway using anti-BTLA mAb (6F7) or C57BL/6-BTLA-/- recipients alone or in combination with CTLA4Ig costimulatory blockade to determine the impact on islet allograft outcome across a fully major histocompatibility complex (MHC)-mismatched barrier. BTLA was upregulated on T lymphocytes after islet transplantation and potentially could be an important target for modulating the allo-response. We demonstrate that islet allograft survival was unaffected by anti-BTLA mAb (6F7) alone, and only slightly enhanced by

CTLA4Ig monotherapy. Paradoxically, when anti-BTLA mAb was combined with CTLA4Ig graft survival was significantly improved. The majority of mice treated with combined costimulatory blockade and coinhibitory modulation achieved long-term (>100d) islet graft survival and accepted donor-specific islet re-transplants. Combined CTLA4Ig and anti-BTLA mAb attenuated initial *in vivo* proliferation as determined by CFSE labeling. Anti-BTLA mAb decreased the BTLA+ lymphocyte numbers, but increased the PD-1+ T cell proportion. And anti-BTLA mAb plus CTLA4Ig-treated mice contained Foxp3+PD-1+ regulatory T (Treg) cells within long-term engrafted islets. Safer immune modulating drugs potentially have benefits for treating both transplant patients and autoimmune diseases.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Adult C57BL/6 (H-2^b), C57BL/6-RAG-KO (B6.129S7-*Rag1*^{tm1mom}/J), BALB/c (H-2^d), and C3H (H-2^k) male mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6-BTLA-/- (BTLA-KO) mice (16) were obtained from Washington University, St. Louis, and bred at the University of Alberta. All mice were housed and cared for in accordance with the guidelines of the Canadian Council on Animal Care.

5.3.2 Diabetes induction and islet transplantation

Recipient C57BL/6 (wild-type, WT) or BTLA-KO mice received a single intraperitoneal (i.p.) injection of streptozotocin (200mg/kg, Sigma-Aldrich, Oakville, Canada) to induce diabetes. Prior to transplantation, diabetes was confirmed by two consecutive blood glucose readings >20mM. Donor BALB/c pancreases were harvested after collagenase ductal infusion (1mg/ml, Sigma-Aldrich). Following mechanical and enzymatic digestion, donor islets were purified using a Ficoll gradient (23, 24) (Sigma-Aldrich). Approximately 500 donor islets were transplanted under the kidney capsule of recipient mice, at 4d post-streptozotocin injection. Allograft function was monitored by serial blood glucose measurements using One-Touch Ultra Glucometer (Johnson & Johnson, New Brunswick, NJ). Successful engraftment was defined as blood glucose level corrected to <8mM by the third day post-transplantation, and graft rejection was defined as blood glucose return to >15mM for two consecutive readings. Graft survival was expressed as median survival time (MST).

5.3.3 *Characterization of BTLA expression on lymphocytes after islet transplantation*

Spleens were recovered from recipient C57BL/6 mice at 2, 5, and 10 days post-transplant. A splenocyte suspension was prepared and stained with anti-BTLA mAb 6F7, anti-B220, anti-TCR, and anti-CD4 for flow cytometric analysis.

5.3.4 *Reagents and treatment protocols*

The anti-mouse BTLA mAb 6F7 (25), CTLA4Ig fusion protein (13), and isotype control IgG1 were purchased from BioExpress Inc. (West Lebanon, New Hampshire). After islet transplantation, recipient mice were treated with monotherapy anti-BTLA mAb, CTLA4Ig (0.25mg i.p., days 0, 2, 4, 6), or IgG1 isotype control; or combined therapy anti-BTLA mAb and CTLA4Ig. All mAbs were administered at 0.50 mg by i.p. injection on day 0, and 0.25 mg on days 2, 4, 6, 8, and 10.

5.3.5 *Confirmation of graft function and re-transplantation*

To test for immunological tolerance, mice with islet allografts surviving long-term (>100d) received a nephrectomy to remove the islet graft-bearing kidney; and, hyperglycemia was confirmed prior to re-challenge with donor specific BALB/c (H-2^d) islet transplants with no further immune modulating treatment.

5.3.6 Reconstitution model of islet allograft rejection

Immunodeficient C57BL/6-RAG-KO mice with chemically-induced diabetes were transplanted with either donor-specific BALB/c or third party C3H islets under the kidney capsule. After engraftment, the immune system was reconstituted with 40 million splenocytes from naïve (C57BL/6) or tolerant (anti-BTLA mAb and CTLA4Ig-treated long-term surviving C57BL/6) mice, and monitored for islet allograft rejection. Results represent n=4 in each group.

5.3.7 CFSE labeling and in vivo T cell proliferation assay

Naïve C57BL/6 splenocytes were labeled with 10μM CFSE (carboxyfluoroscein succinimidyl ester, Molecular Probes, Eugene, OR) as responders. Thirty million CFSE-labeled lymphocytes were adoptively transferred to lethally irradiated (1800 Rad) BALB/c hosts. The mice subsequently received: (i) no treatment, (ii) CTLA4Ig (0.25mg/d), (iii) anti-BTLA mAb (0.25mg/d), or (iv) anti-BTLA mAb and CTLA4Ig. In addition, C57BL/6 CFSE-labeled splenocytes were transferred into syngeneic C57BL/6-RAG-KO mice. The spleens were recovered and stained at 72h with anti-TCR, anti-CD4, and anti-CD8 for four-color flow cytometric analysis. The precursor frequency was calculated, as previously described (26), for T cells that underwent four or more divisions. The peaks were identified according to the number of T cell divisions (n). Since an individual precursor T cell that divides n times generates 2^n daughter cells, the number of precursor for each peak was calculated by dividing the number of daughter cells or events in the n^{th} peak by 2^n. The precursor frequency was then calculated by dividing the sum of the precursor numbers for cells undergoing four or more (to a maximum of 8) divisions by the total number of precursors.

5.3.8 Mixed lymphocyte reaction (MLR)

Responder cells were prepared from the spleens of tolerant and naïve C57BL/6 mice and plated in triplicate wells containing 2×10^5 responder cells with increasing concentrations of irradiated (1500 Rad) stimulator cells derived from C57BL/6 (syngeneic), BALB/c (donor), or C3H (third party) mice. The cells were incubated at 37°C for 72h, then pulsed for 18h with 1μCi ^{3}H-thymidine/well, and harvested for determination of thymidine incorporation, which reflects proliferation.

5.3.9 Cytotoxic T lymphocyte (CTL) assay

The cytotoxic lymphocyte responses were evaluated with the JAM Test as described previously (27). Briefly, 5×10^6 splenocytes from tolerant and naïve C57BL/6 responders were stimulated for 5d with 2×10^6 irradiated BALB/c (donor) or C3H (third party) stimulator splenocytes to expand allospecific T lymphocytes. Con A blast targets were created 40h prior to the CTL assay by culturing 1.5×10^6 naïve syngeneic, donor, and third party splenocytes with Con A (1.25 μg/ml). Before the start of the CTL assay, the blast cells were pulsed with 10μCi of ^{3}H-thymidine for 3h. Responders and targets were then plated at increasing responder to target ratios for 3.5h, before determination of target lysis, which reflects cytotoxic T cell killing.

5.3.10 Adoptive transfer to monitor for cellular depletion

After 30 million splenocytes were adoptively transferred to C57BL/6-RAG-KO mice, they received either isotype control, or anti-BTLA mAb at 0.5mg on day 0 and

0.25mg on days 2, 4, 6, 8 and 10. Following treatment, splenocytes were recovered and absolute lymphocyte numbers were determined using a hemocytometer.

5.3.11 *Immunohistopathology*

Islet allografts were harvested from tolerant (>100 d survival) and control (acutely rejecting) mice. Paraffin sections of formalin fixed samples were stained with hematoxylin and eosin, and for insulin staining using immunoperoxidase, as described (28).

At day 11 post-islet transplantation, after no treatment, anti-BTLA mAb alone, or anti-BTLA mAb 6F7 and CTLA4Ig islet allografts were harvested for immunohistopathology. Cryostat sections were stained using commercial mAbs (eBioscience) to murine CD4, CD8, BTLA, Foxp3 and PD-1 and an Envision immunoperoxidase kit (Dako) (29).

5.3.12 *Flow cytometric analysis*

Splenocyte suspensions of 10^6 cells were blocked, and then stained with labeled antibodies. All staining antibodies were purchased from eBioscience (San Diego, CA, USA) and staining performed according to manufactures recommendations. Stained cells were analyzed using CellQuest software on a FACSCalibur flow cytometer (Becton-Dickson, Braintree, MA, USA).

5.3.13 *Statistical analysis*

Survival curves were analyzed using the Kaplan-Meier method, and graft survival was compared to controls using the log-rank test. Student's t-test was used to analyze two independent groups and a one- or two-way analysis of variance (ANOVA) was used to analyze multiple groups. A p-value <0.05 was considered significant (Prism version 4.0a, GraphPad Software Inc., San Diego, CA, USA).

5.4 RESULTS

5.4.1 BTLA expression is upregulated on T lymphocytes after islet transplantation

To determine the expression of BTLA on lymphocytes after allogeneic challenge, spleens were recovered from chemically-induced diabetic C57BL/6 recipient mice on day 2, 5 and 10 after islet allotransplantation (FIGURE 5-1). BTLA expression increased on T cells upon antigenic stimulation, peaked on day 5 after transplantation ($p<0.05$), but returned to pre-transplant levels by day 10. A representative plot gated on T cells demonstrated higher BTLA expression (38.7%) on day 5 post-transplant compared to (22.2%) naïve splenocytes.

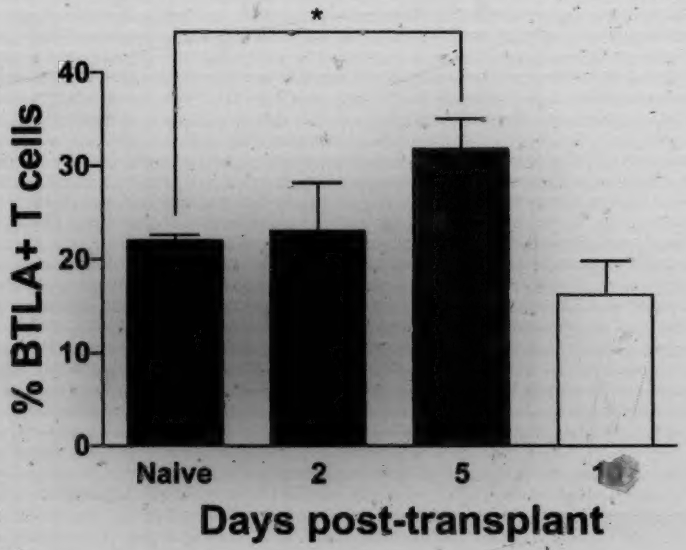


Figure 5-1

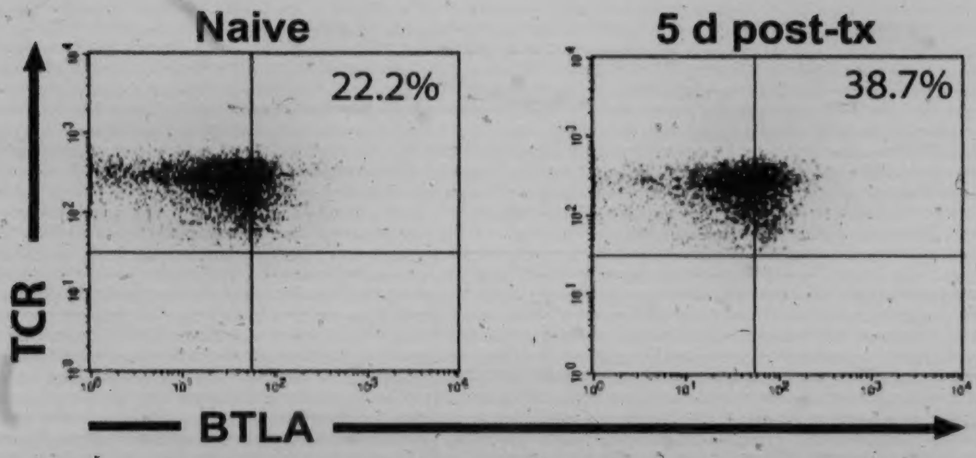
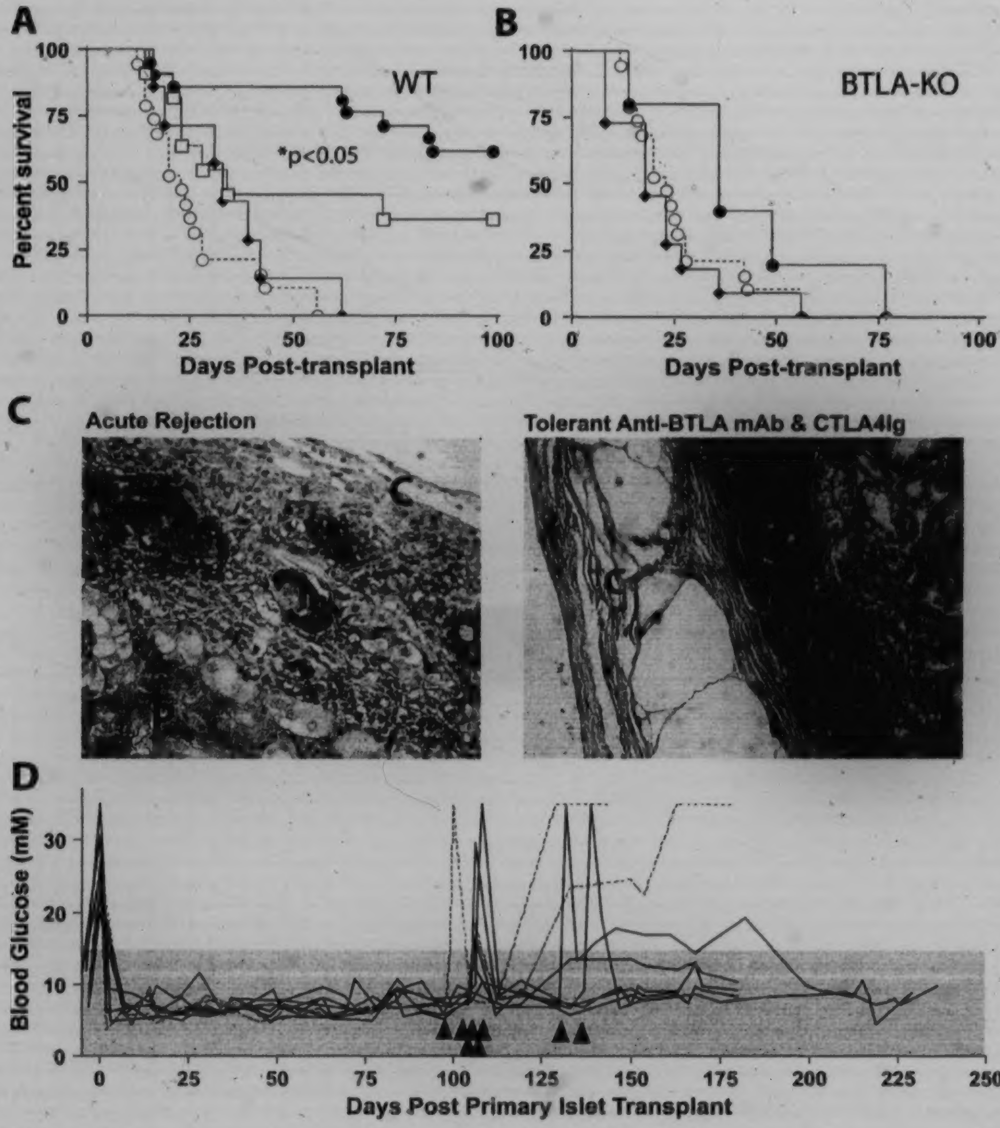


Figure 5-1 BTLA expression increases on T lymphocytes after islet transplantation With no treatment after fully-MHC mismatched islet transplantation, the expression of BTLA was determined by flow cytometry on recipient splenocytes on day 2, 5, and 10 (n=4 at each time point). Representative two-color flow cytometry plot of naïve versus allograft recipient 5 days post-islet transplant, gated on T cells indicating BTLA expression on splenocytes.

5.4.2 Combined anti-BTLA mAb and CTLA4Ig facilitated indefinite islet allograft survival

Since BTLA expression increases after transplantation, the BTLA co-inhibitory pathway may be important in regulating graft rejection, we began by testing anti-BTLA mAb and CTLA4Ig in an islet allograft model (FIGURE 5-2A). Renal subcapsular islet transplants were completed across a strong MHC (H-2^d to H-2^b) and multiple minor-H barriers in mice (BALB/c into C57BL/6 (WT), or BTLA-KO). Control-treated mice rejected islet allografts with no long-term survival (MST=23d), which was reflected by massive lymphocyte infiltration and islet destruction on histology (FIGURE 5-2C). CTLA4Ig monotherapy significantly prolonged graft survival (MST=34d, *$p<0.01$), and was marginally effective in inducing indefinite graft acceptance (>100d) (FIGURE 5-2A). Targeting the BTLA pathway alone using anti-BTLA mAb delayed graft rejection (MST=33d) compared to control, although the difference was not statistically significant. Combined CTLA4Ig and anti-BTLA mAb treatment significantly enhanced islet allograft survival compared to CTLA4Ig alone (*$p<0.05$), and long-term engrafted islets were free of significant lymphocyte infiltrate, with strong insulin staining on histology (FIGURE 5-2C). BTLA-KO recipients did not enhance islet allograft rejection or survival (MST=18d), and when BTLA-KO recipients were treated with CTLA4Ig, islet graft rejection was delayed (MST=36d) compared to control-treated WT mice (MST=23d), however the difference was not statistically significant (FIGURE 5-2B).



Acute Rejection

Tolerant Anti-BTLA mAb & CTLA4Ig

Figure 5-2

Figure 5-2 Islet allograft survival and non-fasting blood glucose levels
Approximately 500 donor (BALB/c) islets were transplanted under the kidney capsule of recipient C57BL/6 (WT) or C57BL/6-BTLA-/- (BTLA-KO) mice with chemically induced diabetes. **(A)** After islet allograft, mice were treated with either isotype control (white circle, n=19), CTLA4Ig alone (white square, n=11), anti-BTLA mAb alone (black diamond, n=7), or combined CTLA4Ig and anti-BTLA mAb treatment (black circle, n=21). **(B)** Islet allograft survival was not enhanced when transplanted into BTLA-KO mice (black diamond, n=11), and the addition of CTLA4Ig prolonged islet allograft survival (black circle, n=5). For comparison only, WT mice treated with isotype control Ab was included (white circle, n=19). **(C)** Representative islet cross-section of an acutely rejecting allograft and of a long-term (>100d) engrafted islet transplant after anti-BTLA mAb and CTLA4Ig treatment (the kidney capsule, islet allograft, and kidney parenchyma are labeled **C**, **I**, **P**, respectively). All sections were stained with hematoxylin, eosin, and for insulin, x400). **(D)** The random blood glucose levels of eight tolerant mice treated with anti-BTLA mAb and CTLA4Ig were followed after allogeneic islet transplant (solid black line represents mice tolerant of second donor-specific graft, and dotted grey lines indicate mice that rejected a second donor-specific graft). Nephrectomy was performed at 100 days in 6 mice and at > 130 days in another 2 mice (arrowheads indicate time of primary graft removal by nephrectomy).

5.4.3 *Anti-BTLA mAb and CTLA4Ig induces donor-specific tolerance in vivo, but not in vitro*

Since anti-BTLA mAb and CTLA4Ig were additive in prolonging islet allograft survival, we next tested for donor-specific tolerance. Recipients with indefinite graft survival were re-challenged with a second islet transplants after removal of the first graft by nephrectomy, or by a reconstitution model *in vivo*.

Thirteen of 21 (61.9%) mice initially treated with anti-BTLA mAb and CTLA4Ig achieved long-term graft survival >100d. Eight tolerant mice underwent nephrectomy to remove the graft-bearing kidney and all mice returned to hyperglycemia confirming graft function (FIGURE 5-2D). In six mice, the primary islet graft was removed at 100d, and in two mice at 130 and 137d, respectively. The random blood glucose levels were stable over the follow-up periods. Six of 8 tolerant mice accepted second donor-specific islet allograft and returned to normoglycemia, without further immune therapy (FIGURE 5-3A).

To explore the tolerant state *in vivo* without hyperglycemia, we used an adoptive transfer model (FIGURE 5-3B). T cells from mice with prolonged graft function treated initially with anti-BTLA mAb and CTLA4Ig did not reject donor-specific islets, maintaining normal blood glucose levels, but were able to reject third party islet grafts, promptly causing a return to hyperglycemia after reconstitution (*p=0.0282).

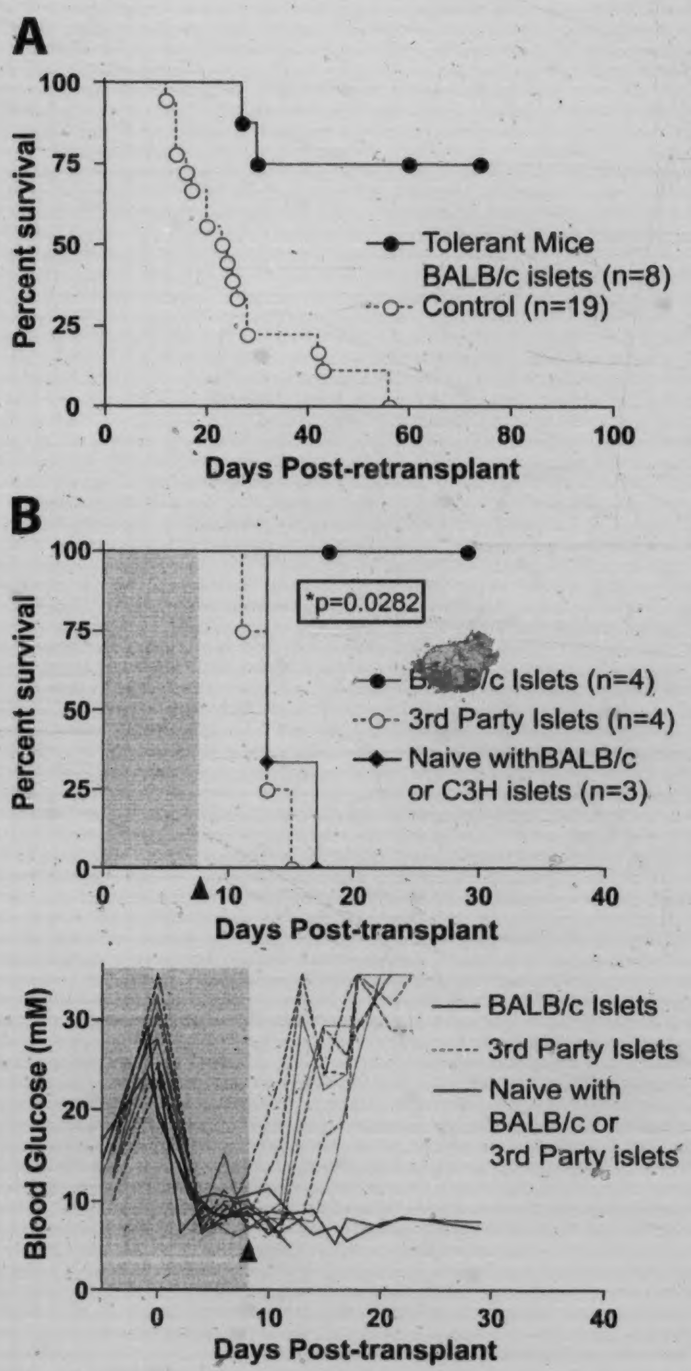

A
Percent survival
100
75
50
25
0
0
20
40
60
80
100
Days Post-retransplant
Tolerant Mice
BALB/c islets (n=8)
Control (n=19)
B
Percent survival
100
75
50
25
0
0
10
20
30
40
Days Post-transplant
*p=0.0282
B[illegible]/c Islets (n=4)
3rd Party Islets (n=4)
Naive withBALB/c
or C3H islets (n=3)
Blood Glucose (mM)
30
20
10
0
0
10
20
30
40
Days Post-transplant
BALB/c Islets
3rd Party Islets
Naive with
BALB/c or
3rd Party islets

Figure 5-3

Figure 5-3 Mice treated with anti-BTLA mAb 6F7 and CTLA4Ig demonstrate donor-specific tolerance *in vivo*

(A) After primary graft removal by nephrectomy, tolerant mice (black circles) were re-challenged with a second donor-specific islet allograft without further immune therapy. Control-treated mice (white circles) are included for comparison purposes only. **(B)** Immunodeficient C57BL/6-RAG-KO mice received either donor-specific BALB/c islets (black circles) or third party C3H islets (white circles). After an engraftment period (shaded area), splenocytes from tolerant (long-term surviving islet grafts >100 d, initially treated with anti-BTLA mAb and CTLA4Ig) or naïve mice were adoptively transferred on day 6 post-transplant (indicated by the arrowhead) to reconstitute the immune system of the graft recipients.

Next we examined the robustness of the tolerance by testing the T cell responses *in vitro* using MLR and CTL (FIGURE 5-4). Splenocytes from 5 tolerant mice treated with anti-BTLA mAb and CTLA4Ig with long-term islet allografts, did not show significantly reduced proliferation in response to donor-specific or third-party antigen as compared to naïve mice. In addition, *in vitro* CTL killing was not different in long-term engrafted mice versus naïve mice.

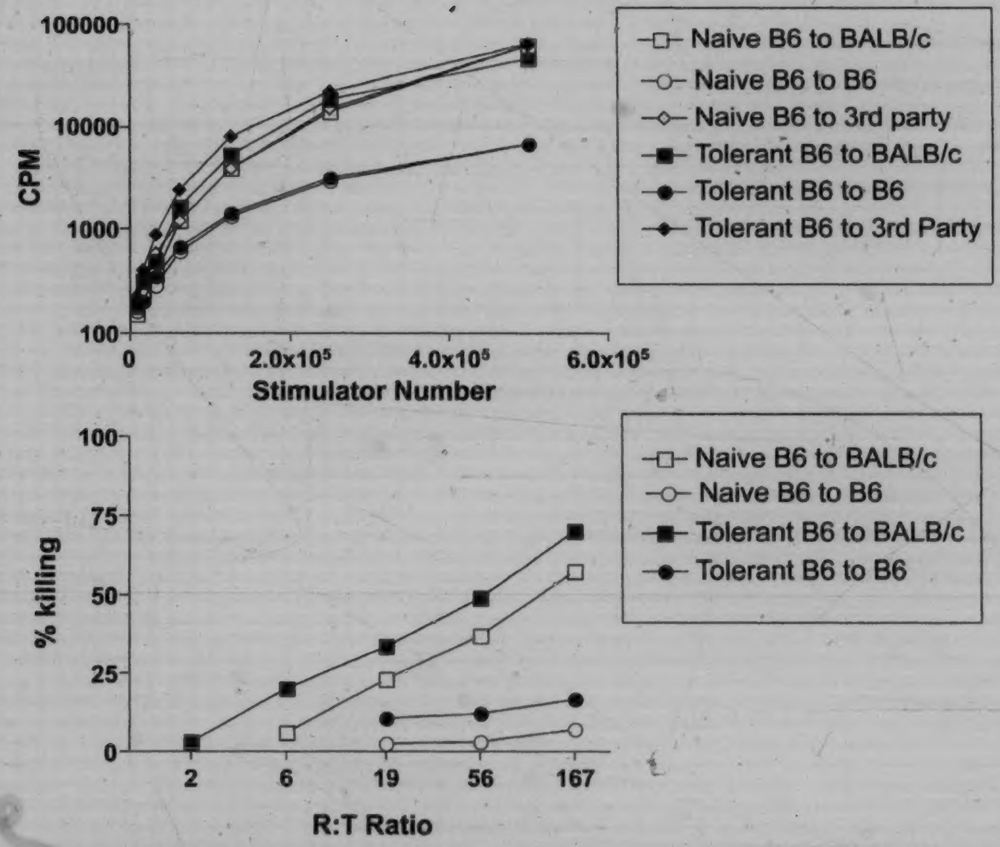


Figure 5-4 Anti-BTLA mAb and CTLA4Ig did not induce *in vitro* allo-specific unresponsiveness

MLRs and CTLs were performed to investigate whether mice, tolerant to islet allografts *in vivo*, could react to allo- and third party-antigen *in vitro*. Tolerant splenocytes proliferated just as strongly to BALB/c stimulus as naïve splenocytes; furthermore, there was no difference in the response to third party antigen. The CTL assay did not reveal any significant difference between naïve and tolerant splenocytes. Results are representative of splenocytes recovered at 100 d after islet transplant from 5 different tolerant mice treated with anti-BTLA mAb and CTLA4Ig.

5.4.4 Anti-BTLA mAb and CTLA4Ig treatment attenuates initial CD4+ and CD8+ T cell alloresponses

CD28 is constitutively expressed on T cells and CTLA4Ig treatment may affect the early immune response. We evaluated whether CTLA4Ig acted by suppressing initial alloresponses and whether combined treatment with anti-BTLA mAb would be synergistic. The *in vivo* CFSE proliferation assay is represented by histograms depicting cellular division (FIGURE 5A & 5B). After transfer to lethally irradiated BALB/c hosts, untreated lymphocytes demonstrated robust T cell alloresponses (FIGURE 5-5A), while T lymphocytes transferred to syngeneic hosts had negligible proliferation (FIGURE 5-5B). Qualitatively, treatment with CTLA4Ig dramatically reduced proliferation in both CD4+ and CD8+ T lymphocytes. Anti-BTLA mAb monotherapy did not reduce cellular division in T cells. However, CTLA4Ig and anti-BTLA mAb treatment demonstrated an additional reduction in proliferation compared to CTLA4Ig alone.

To quantitate *in vivo* proliferation, the T cell percent precursor frequency was calculated (FIGURE 5-5C). T and CD4+ T cell proliferation was significantly decreased after CTLA4Ig treatment (*$p<0.05$). Although anti-BTLA mAb alone does not limit initial T cell proliferation, the addition of anti-BTLA mAb to CTLA4Ig further enhanced the reduction in precursor frequency (**$p<0.01$).

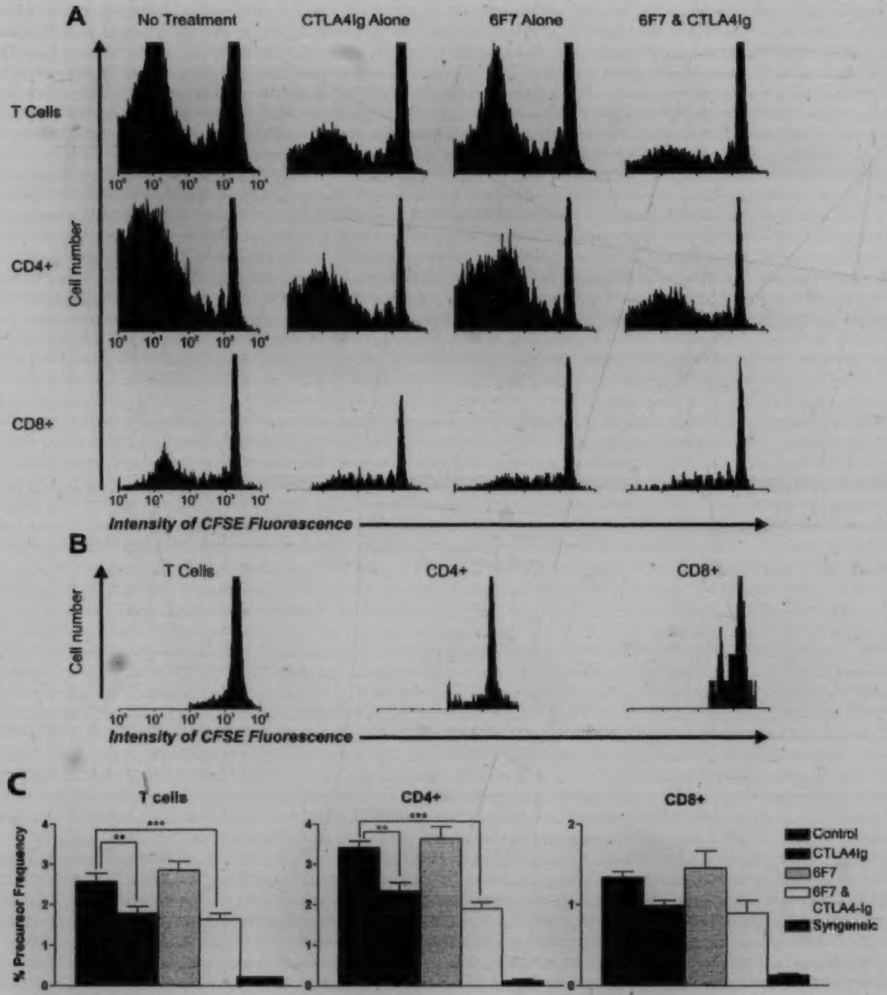


Figure 5-5 CFSE in vivo allo-specific T cell proliferation was limited by CTLA4Ig and combined anti-BTLA mAb and CTLA4Ig treatment

Naïve splenocytes were labeled with fluorescent dye CFSE before adoptive transfer into supralethally irradiated BALB/c (**A**) or syngeneic C57BL/6-RAG-KO (**B**) mice. Mice were then treated with (n=7 in each group): (i) no treatment, (ii), CTLA4Ig alone, (iii), anti-BTLA mAb alone, (iv), anti-BTLA mAb + CTLA4Ig, and (v) syngeneic control. Splenocytes were harvested 72 h after adoptive transfer and analyzed by flow cytometry for CD8 and CD4 expression. The peak on the far right represents undivided cells, and subsequent peaks with decreasing fluorescent intensity represent successive divisions. **C.** The precursor frequency was calculated for lymphocytes, which underwent four or more (to a maximum of 8) divisions for each treatment group. Combined treatment was most effective in limiting the initial allospecific T cell response.

5.4.5 Anti-BTLA mAb reduced BTLA+ lymphocyte number, while PD-1 expression significantly increased

To examine the mechanism in which anti-BTLA mAb may enhance islet allograft survival, we evaluated lymphocyte number and coinhibitory receptor expression after adoptive transfer of approximately 30 million C57BL/6 splenocytes into C57BL/6-RAG-KO recipients. The BTLA expression on lymphocytes was significantly reduced after anti-BTLA mAb treatment compared to controls (*$p<0.05$), and the absolute number of total T, CD4+ T, and B cells were significantly lower than controls (*$p<0.05$) (FIGURE 5-6A). Furthermore, the percent T and CD4+ T cells expressing PD-1 were significantly increased after anti-BTLA mAb treatment compared to control (*$p<0.05$) (FIGURE 5-6B).

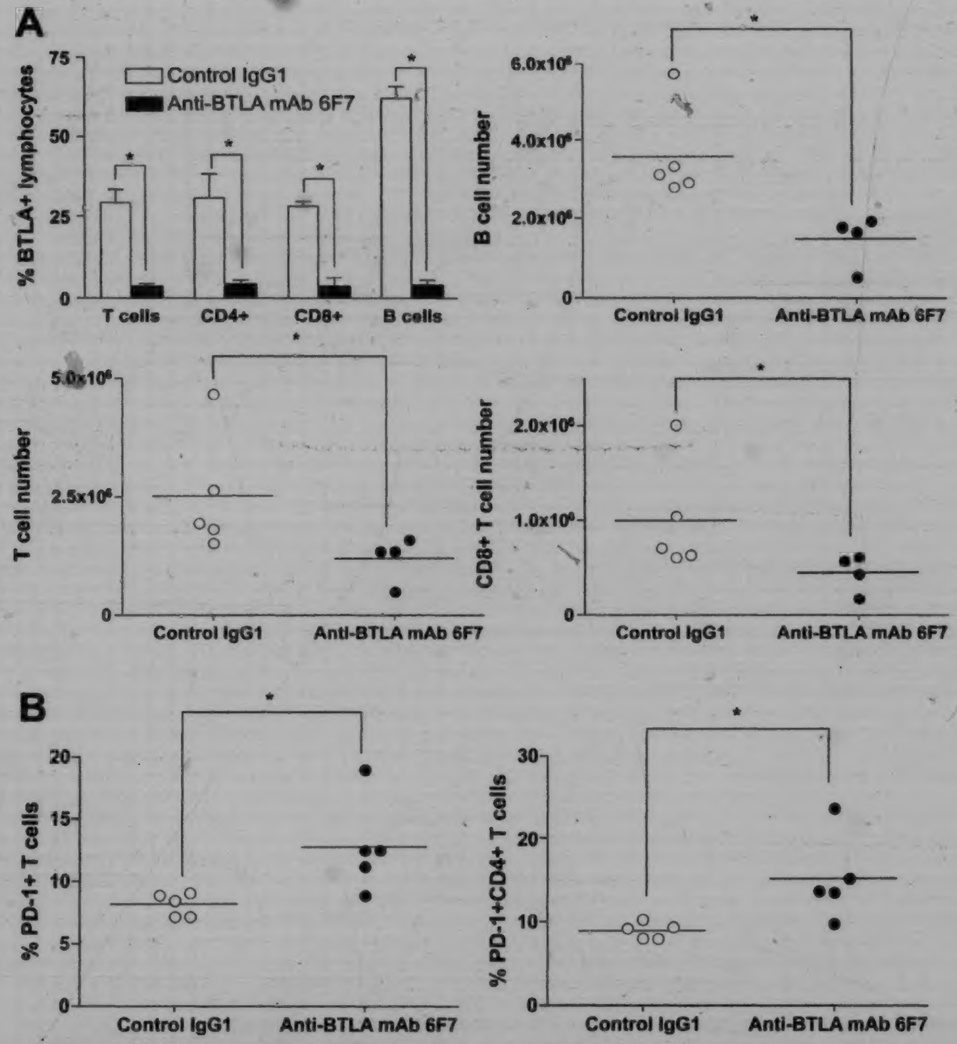


Figure 5-6 Anti-BTLA mAb decreased BTLA+ lymphocyte numbers and increased the proportion of PD-1 expressing T cells

After adoptive transfer of C57BL/6 splenocytes to C57BL/6-RAG-KO recipients, mice were treated with either anti-BTLA mAb or isotype control IgG1. The absolute lymphocyte numbers were determined by hemocytometer and coinhibitory receptors BTLA and PD-1 expression was determined by flow cytometry. The percentages represent proportion of T lymphocytes that were PD-1+ or CD4+PD-1+. Results are representative of two different experiments with n=5 in each group.

5.4.6 Local accumulation of regulatory T cells

Serial sections of subcapsular islet allografts, harvested at 11 d post-islet transplant in no treatment control recipients, were infiltrated by CD4 cells, CD8 cells, and cells expressing BTLA and PD-1, but little or no Foxp3 (FIGURE 5-7). In contrast, islet allografts in recipients treated with anti-BTLA mAb plus CTLA4Ig showed well-preserved islets with peri-islet accumulation of CD4+ and CD8+ cells, expression of BTLA and PD-1, and a prominent accumulation of Foxp3+ host mononuclear cells.

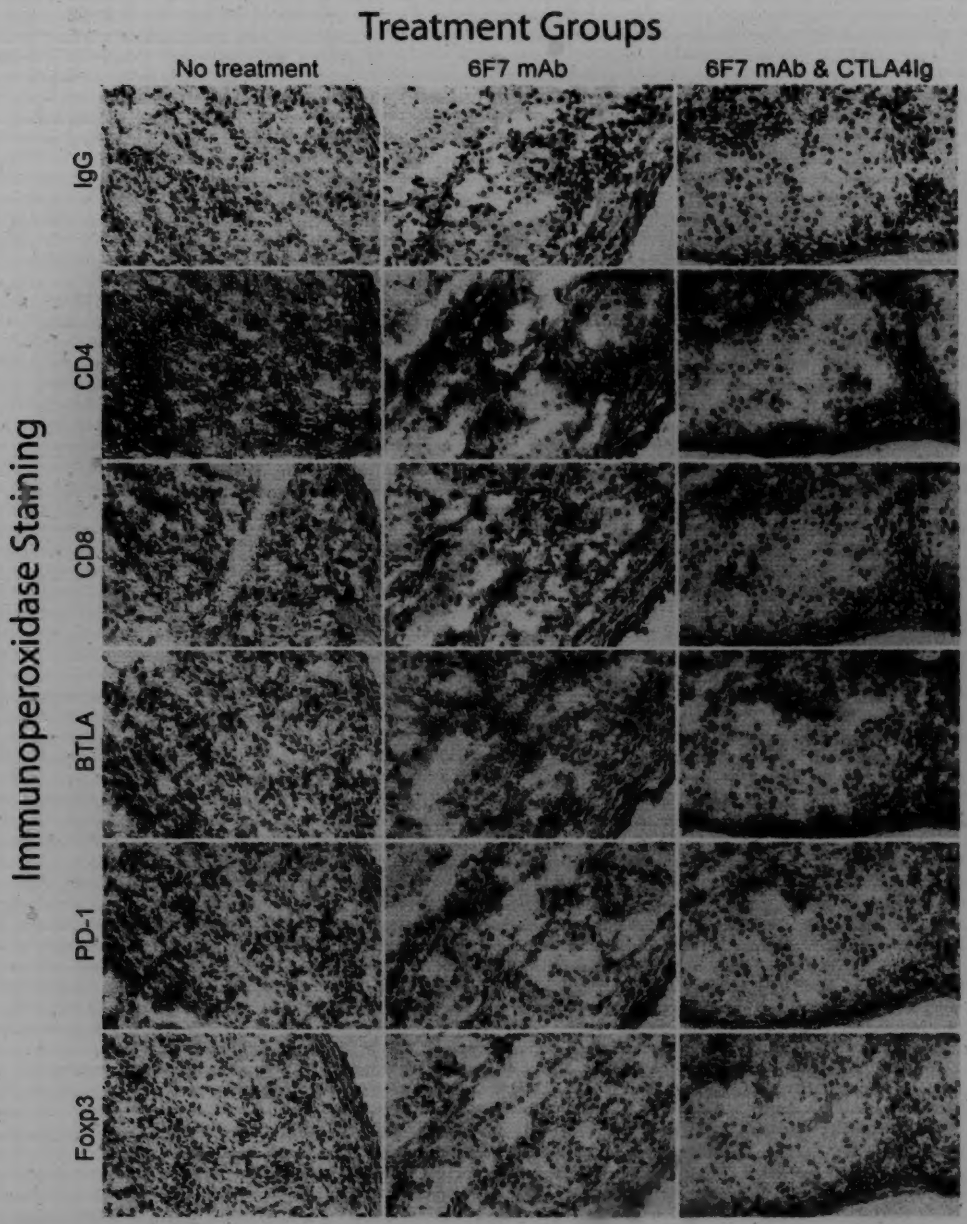

Treatment Groups
No treatment
6F7 mAb
6F7 mAb & CTLA4Ig
Immunoperoxidase Staining
IgG
CD4
CD8
BTLA
PD-1
Foxp3

Figure 5-7 Foxp3+ cell recruitment to islet allografts in mice treated with anti-BTLA mAb and CTLA4Ig

Fully MHC-mismatched islet allografts were transplanted under the kidney capsule and recipients were treated with control IgG1 (no treatment), anti-BTLA mAb alone (6F7), or combination treatment 6F7 and CTLA4Ig. After 11 days of treatment the allografts were harvested for cryostat sectioning and immunoperoxidase staining using mAbs to CD4, CD8, BTLA, Foxp3 and PD-1, as well as control IgG. Positive cells are stained red/brown color. (Hematoxylin-stained cryostat sections, original magnifications x300).

5.5 DISCUSSION

The discovery of coinhibitory pathways implies that the balance between negative and positive co-signaling determines the fate of the immune response. As a result, manipulation of one pathway may not be enough to induce tolerance towards alloantigen. The strategy of targeting the coinhibitory pathway may improve allograft survival. As proof of principle, this approach has been shown to be potentially very useful in the context of suboptimal costimulation. The PD-L1Ig fusion protein can prolong fully MHC-mismatched cardiac grafts in CD28-deficient mice (19), and when PD-L1Ig is combined with anti-CD154 islet allograft survival is markedly increased (18). We hypothesized that the BTLA coinhibitory pathway may be an important target in transplantation, since BTLA blockade has been reported to alter cardiac graft survival (22). To determine whether the BTLA coinhibitory pathway may play a role in islet allotransplantation rejection or tolerance we tested the hypothesis in the fully MHC-mismatched islet transplantation mouse model. In addition, we investigated whether anti-BTLA mAb could interact with costimulatory blockade and potentially affect islet graft survival.

We first examined the expression pattern of BTLA in islet allografts at 2, 5, and 10 days. BTLA was significantly upregulated on T cells after alloantigen challenge *in vivo*. BTLA expression on T lymphocytes peaked at day 5 and was back to normal levels by day 10. Therefore targeting the BTLA pathway may be most effective during the first 10 days post-transplant.

Monotherapy targeting the BTLA pathway with mAb did not accelerate islet allograft rejection, unexpectedly, anti-BTLA mAb 6F7 modestly delayed rejection compared to controls, although this did not reach statistical significance. Consistent with a previous report, costimulatory blockade with CTLA4Ig marginally enhanced islet allograft survival (30). However, in the context of CTLA4Ig costimulatory blockade, BTLA-specific mAb paradoxically induced long-term (>100d) islet allograft survival (*p<0.05). In contrast, BTLA-KO recipients treated with CTLA4Ig demonstrated only slightly increased islet allograft survival compared to control, and did not reach statistical significance. A recent study demonstrated that the BTLA-deficient mice had increased numbers of memory-phenotype CD8$^+$ T cells compared to wild-type mice, leading to hyperproliferative responses *in vitro* (31). Classical (B7:CD28) costimulation is less important for memory T cells (32, 33) and CD8+ T cells (34), both of which may mediate allograft loss. Therefore, BTLA-deficient mice may be less susceptible to CTLA4Ig costimulatory blockade compared to WT mice. The results also suggest that BTLA-specific mAb was not blocking the BTLA negative signal, and may have a different mechanism of action.

To further evaluate the state of tolerance, we re-challenged the tolerant mice with a second donor-specific islet graft and by a reconstitution model. Without further immune modulation, the majority of tolerant mice (initially treated with anti-BTLA mAb and CTLA4Ig) accepted the second donor-specific (BALB/c) islet allograft. We were unable to rechallenge with third party grafts as this would have rendered these mice anephric.

We therefore tested for third-party responsiveness in the adoptive transfer model, and clearly demonstrated rapid rejection of third-party (C3H) grafts (n=4) but no rejection of the donor-specific BALB/c islets (n=4). Adoptive transfer may result in homeostatic proliferation, but this did not prevent the acceptance of donor-specific BALB/c islets within the time frame analyzed. T cells from mice with indefinite graft survival initially treated with anti-BTLA mAb and CTLA4Ig were able to reject third-party but accepted donor-specific islet grafts, indicating donor-specific immunological tolerance. The tolerance in the adoptive transfer model also suggested that diabetes-induced immunosuppression, which can be a confounding variable when second donor islet transplants are given to hyperglycemic recipients (35), was not a major factor contributing to donor islet graft acceptance (T cells were transferred into normoglycemic recipients).

In contrast, we found that the tolerant mice treated with anti-BTLA mAb and CTLA4Ig had intact allo-specific proliferative and cytotoxic responses *in vitro*. New allo-specific thymic emigrants may account for reactivity *in vitro*, but the absence of appropriate co-signals within an established graft may not induce rejection *in vivo* (36). However, mice were tolerant to the second transplants without costimulation blockade *in vivo*, therefore an alternative explanation is that they react to different antigens *in vitro*. The *in vitro* assays primarily present donor antigens in the context of splenic dendritic antigen presenting cells (APC), which may be different from the antigens encountered *in vivo*, where islet antigen predominates. We have experimentally demonstrated this concept in a previous study, when comparing responses to MHC on antigen presenting cells vs. responses to the same MHC expressed by a thymus transplant (37).

An *in vivo* proliferation CFSE assay was used to evaluate the immunological mechanism of improved graft survival in mice treated with anti-BTLA mAb and CTLA4Ig. CD28 is constitutively expressed on T cells (9) and the survival benefits observed with CTLA4Ig may be attributed to the effects on T lymphocytes immediately after transplantation. This rationale was supported by a marked reduction in T cell division and precursor frequency in the CFSE *in vivo* proliferation assay carried out in the first 72 hours. The addition of anti-BTLA mAb to CTLA4Ig enhanced the reduction in proliferation compared to control, which suggests that anti-BTLA mAb decreased alloreactive T cell number or activation. *In vitro* T cell stimulation with anti-CD3 mAb was not inhibited by the presence of anti-BTLA mAb (data not shown) and argues against agonistic activity at the BTLA receptor.

To examine the effects of anti-BTLA mAb on lymphocyte populations and coinhibitory receptor expression, we used an adoptive transfer model to monitor lymphocytes in the absence of new thymic and bone marrow emigrants. BTLA expressing lymphocytes were significantly reduced after anti-BTLA mAb treatment, and T and B cell numbers were significantly reduced. Furthermore, anti-BTLA mAb increased the proportion of PD-1+ and PD-1+CD4+ T cells compared to control, which may result in increased PD-1 negative co-signaling and enhanced allograft protection (22). Long-term engrafted islets from mice treated with anti-BTLA mAb and CTLA4Ig revealed intact islets with positive insulin staining and a paucity of mononuclear lymphocytes at the edge of the graft. Similarly, islet allografts from mice treated with anti-BTLA mAb and CTLA4Ig, harvested 11 days after transplantation, showed healthy islets with peri-islet accumulation of CD4+ and CD8+ cells, expression of BTLA and PD-1, and prominent Foxp3+ cells, perhaps conferring protection by a regulatory role. In contrast, at 11 days post-transplant, untreated mice had islet allografts with significant infiltration by CD4 cells, CD8 cells, and cells expressing BTLA and PD-1, but little or no Foxp3 cells.

In summary, we report that the combination of anti-BTLA mAb and CTLA4Ig prolonged islet allograft survival and promoted indefinite graft acceptance. Anti-BTLA mAb and CTLA4Ig therapy induces a donor-specific tolerant state in mice. We demonstrate that tolerant mice accept second donor-specific islet transplants without additional immune modulating intervention. Furthermore, tolerant splenocytes rejected third party islets, and accepted donor-specific islet allografts *in vivo*. CTLA4Ig acts, in part, to limit the initial proliferation of allogeneic lymphocytes, and the addition of an anti-BTLA mAb potentiated the effects of CTLA4Ig, by depletion of pathogenic BTLA+ lymphocytes. To investigate this possibility, a separate series of experiments are required to determine the impact of BTLA depletion on the donor-specific T lymphocytes and cytokine environment. BTLA is expressed on terminally differentiated T helper (T_H)-1 cells (16); therefore, depletion of BTLA+ cells may shift the cytokine balance towards a T_H2 environment, which is more conducive to Treg formation. Our data supports this hypothesis, since islet allografts from mice treated with anti-BTLA mAb and CTLA4Ig contained CD4 cells, CD8 cells, BTLA and PD-1 expressing cells, and prominent Foxp3 cells, which may be acting as regulatory cells within the graft. In addition, anti-BTLA mAb may act through upregulation of PD-1 negative co-signaling (22). We speculate there may be a specific pathway for PD-1 induction that is inhibited by BTLA engagement. Taken together anti-BTLA mAb with CTLA4Ig costimulatory blockade was additive in prolonging donor specific tolerance of islet allografts in mice, and may be a useful adjunctive strategy for promoting donor-specific tolerance in transplantation in the future.

5.6 ACKNOWLEDGMENTS

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CHAPTER SIX

NEGATIVE AND POSITIVE COSIGNALING WITH ANTI-BTLA (PJ196) AND CTLA4Ig PROLONGS ISLET ALLOGRAFT SURVIVAL

NOTE: A previous version of this chapter was accepted for publication in Transplantation and is currently in press. The authors on this paper include: Truong W, Plester JC, Hancock WW, Kaye JG, Merani S, Murphy KM, Murphy TL, Anderson CC, and Shapiro AMJ.

6.1 OVERVIEW

The novel coinhibitory receptor B and T lymphocyte attenuator (BTLA) has been implicated in the regulation of autoimmune and may potentially play a role in alloimmune responses. An anti-BTLA mAb has been reported to prolong fully MHC-mismatched cardiac allograft survival, and we test here the hypothesis that anti-BTLA mAb PJ196 may synergize with CTLA4Ig costimulatory blockade in islet transplantation. We investigated the potential of PJ196, and show that it did not deplete BTLA expressing cells, but it caused down-regulation of BTLA on the surface of lymphocytes and accumulation of cells with regulatory phenotype at the graft site, promoting islet allograft acceptance together with CTLA4Ig. The combination of BTLA coinhibitory modulation and CTLA4Ig costimulatory blockade may be an effective adjunctive strategy for inducing long-term allograft survival.

6.2 INTRODUCTION

Despite recent advances in islet transplantation, chronic immunosuppression is still required to prevent allograft rejection, and therefore limits the broad application of the procedure in patients with type 1 diabetes mellitus (*1*). Alloreactive T lymphocyte proliferation plays a critical role in islet graft rejection, and optimal activation requires a primary antigen-specific T cell signal along with a secondary co-signal (*2*). A myriad of coordinated positive and negative co-signaling receptors direct lymphocytes towards either activation or tolerance (*3*). Targeting co-signaling receptors may be effective adjuncts to existing immune therapy or an independent strategy to promote graft tolerance and avoid toxic immunosuppressive drugs.

The negative co-signaling molecule B and T lymphocyte attenuator (BTLA) (*4*) is the newest member of the immunoglobulin superfamily, which includes coinhibitory receptors cytotoxic T lymphocyte antigen-4 (CTLA4) (*5*), and programmed cell death-1 (PD-1) (*6*). The most well studied positive and negative co-signaling pair, CD28 (*7*) and CTLA4 (*8*), share ligands B7-1 and B7-2. The recombinant fusion protein CTLA-4-immunoglobulin (CTLA4Ig) competitively blocks CD28:B7-1/B7-2 costimulatory binding (*9*). CTLA4Ig has been used successfully in various allograft and xenograft animal models (*10-12*), but alone is only marginally effective in prolonging islet allograft survival (*13*). Coinhibitory pathway targeting potentially provides a novel approach to transplantation tolerance. The fusion protein PD ligand-1 immunoglobulin (PDL1Ig) plus rapamycin induced permanent survival in fully MHC-mismatched cardiac allografts (*14*). Since anti-BTLA mAb has been reported to modulate cardiac graft survival (*15*), we have begun to test the hypothesis that anti-BTLA mAb may synergize with CTLA4Ig costimulatory blockade in islet allografts.

Murine BTLA has structural and expression polymorphisms (*16*), and allelic variations exist among laboratory mouse strains. Anti-BTLA mAb clone 6F7 recognizes BTLA from both C57BL/6-like and BALB/c-like alleles (*16*). The independently described mAb clone PJ196 only binds C57BL/6-like BTLA (*17*). We recently demonstrated in a separate study that the anti-BTLA mAb, clone 6F7, *in vivo* depletes alloaggressive BTLA expressing cells and potentiates costimulation blockade in promoting islet allograft tolerance and attenuated autoimmune disease precipitated by anti-PD-1 blockade in NOD mice (submitted manuscripts). The depleting anti-BTLA mAb provides evidence to support the role of BTLA expression on pathogenic lymphocytes, which suggests that the BTLA pathway is important for negatively regulating both autoimmune diabetes and allograft rejection on these cells. This is consistent with previous studies showing the preferential expression of BTLA on terminally differentiated pathogenic T helper 1 (T_H1) cells and the lack of expression on tolerogenic T_H2 cells (*4*). However, a non-depleting anti-BTLA mAb may provide information on the direct role of BTLA on lymphocyte activation or regulation. In this study, we test the hypothesis that a non-depleting anti-BTLA mAb, may affect allograft acceptance. Herein, we investigate the potential of the non-depleting anti-BTLA mAb, clone PJ196, and demonstrate that it does not deplete BTLA expressing cells, but nevertheless promotes islet allograft acceptance together with CTLA4Ig.

6.3 METHODS & RESULTS

6.3.1 Combination anti-BTLA mAb & CTLA4Ig facilitates long-term islet allograft survival

To investigate the impact of targeting the BTLA pathway with PJ196 in islet transplantation, we began by evaluating fully MHC-mismatched BALB/c (H-2^d) donor islets transplanted into C57BL/6 (H-2^b) recipients that were either wild-type (WT, from

Jackson Laboratory, Bar Harbor, ME, USA) or C57BL/6-BTLA-/- (BTLA-KO) mice (*4*) (FIGURE 6-1). All mice were housed and cared for in accordance with the guidelines of the Canadian Council on Animal Care. Recipients with streptozotocin-induced diabetes were transplanted with approximately 500 purified donor islets under the renal capsule as previously described (*18, 19*).

Following transplantation, recipient mice were treated with reagents by intraperitoneal injection. Anti-BTLA mAb (mouse IgG1, clone PJ196; BioExpress Inc., West Lebanon, New Hampshire) (*16*) and isotype control were administered at 0.50mg on day 0, and 0.25mg on days 2, 4, 6, 8, and 10 post-transplant. CTLA4Ig (BioExpress) (*20*) was administered at 0.25mg on days 0, 2, 4, and 6 days after transplantation. Anti-BTLA mAb PJ196 or CTLA4Ig, as monotherapy significantly prolonged graft survival ($p<0.02$) compared to control, but the majority eventually rejected (FIGURE 6-1).

CTLA4Ig acts by blocking CD28 costimulation signaling thus limiting the initial expansion of allogeneic T lymphocytes. Anti-BTLA mAb treatment significantly enhanced graft survival induced by CTLA4Ig alone ($p<0.05$), and the majority of islet allografts achieved long-term (>100d) survival (FIGURE 6-1).

Anti-BTLA mAb PJ196 does not appear to behave simply as an agent that blocks BTLA on pathogenic effector cells, since blockade of a negative cosignaling receptor on such cells would be expected to accelerate rejection. Instead PJ196 prolongs islet allograft survival and potentiates the effects of CTLA4Ig. An alternative possibility is that PJ196 inhibits islet rejection due to an agonistic property that triggers the BTLA coinhibitory signal. A previous study showed that PJ196 did not decrease *in vitro* T cell proliferation, arguing against mAb agonistic activity on the negative BTLA co-receptor (*17*).

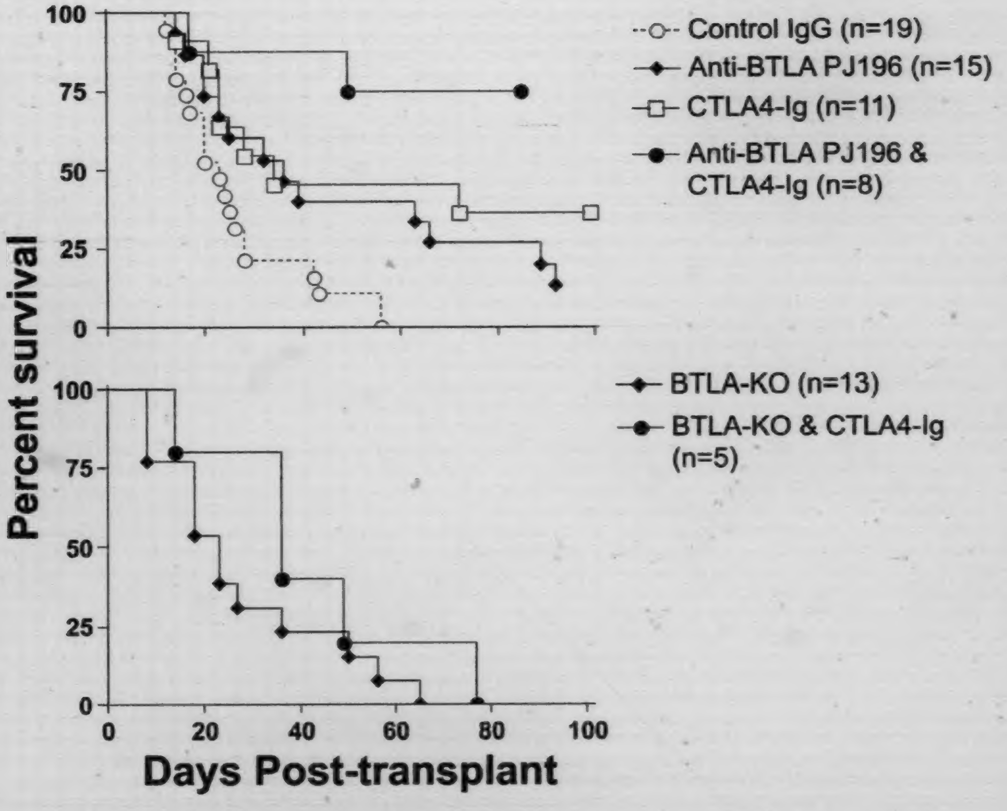

Figure 6-1 Anti-BTLA mAb PJ196 and CTLA4Ig facilitates long-term islet allograft survival

Approximately 500 BALB/c islets were transplanted in the renal sub-capsule across a strong MHC-mismatched barrier ($H\text{-}2^d$ to $H\text{-}2^b$) into wild-type or BTLA-KO, C57BL/6 recipients. Graft survival was expressed as median survival time (MST). Long-term survival was defined as islet grafts surviving >100d. Monotherapy using anti-BTLA mAb PJ196 (MST=36d, $p<0.02$) delayed islet allograft rejection compared to control (MST=20d); however, the majority of grafts eventually failed. CTLA4Ig significantly potentiated the effects of anti-BTLA mAb in promoting allograft survival ($p<0.05$), and the majority of mice achieved long-term (>100d) graft survival. The Kaplan-Meier method was used to analyze graft survival, and the log-rank test was used for comparisons between treatment groups.

6.3.2 Anti-BTLA mAb PJ196 decreases the surface expression of BTLA, but does not cause depletion of on B and T lymphocytes

To determine the mechanism of anti-BTLA mAb PJ196, we examined its effects on lymphocyte populations systemically and locally at the allograft site. We began by characterizing lymphocytes harvested from C57BL/6 mice treated with two 0.10mg doses of either anti-BTLA mAb or isotype control. Lymphocytes were stained *in vitro* with either fluorescently labeled secondary antibody alone or with anti-BTLA mAb PJ196 plus labeled secondary antibody. There was no change in the T or B cell populations and the relative proportions of CD4+ and CD8+ T cells remain the same between anti-BTLA mAb-treated and control mice (FIGURE 6-2A). BTLA expression is most intense on B cells followed by CD4+, and then CD8+ T cells. After anti-BTLA mAb treatment, not all BTLA receptors were saturated with antibody *in vivo* since additional anti-BTLA mAb *in vitro* with secondary Ab staining increases the fluorescence. Anti-BTLA mAb caused significant (3 to 5 fold) down-regulation of BTLA on the surface of both B and T cells, since saturating staining after *in vivo* anti-BTLA mAb injection never reaches the level of fluorescence observed in control mice.

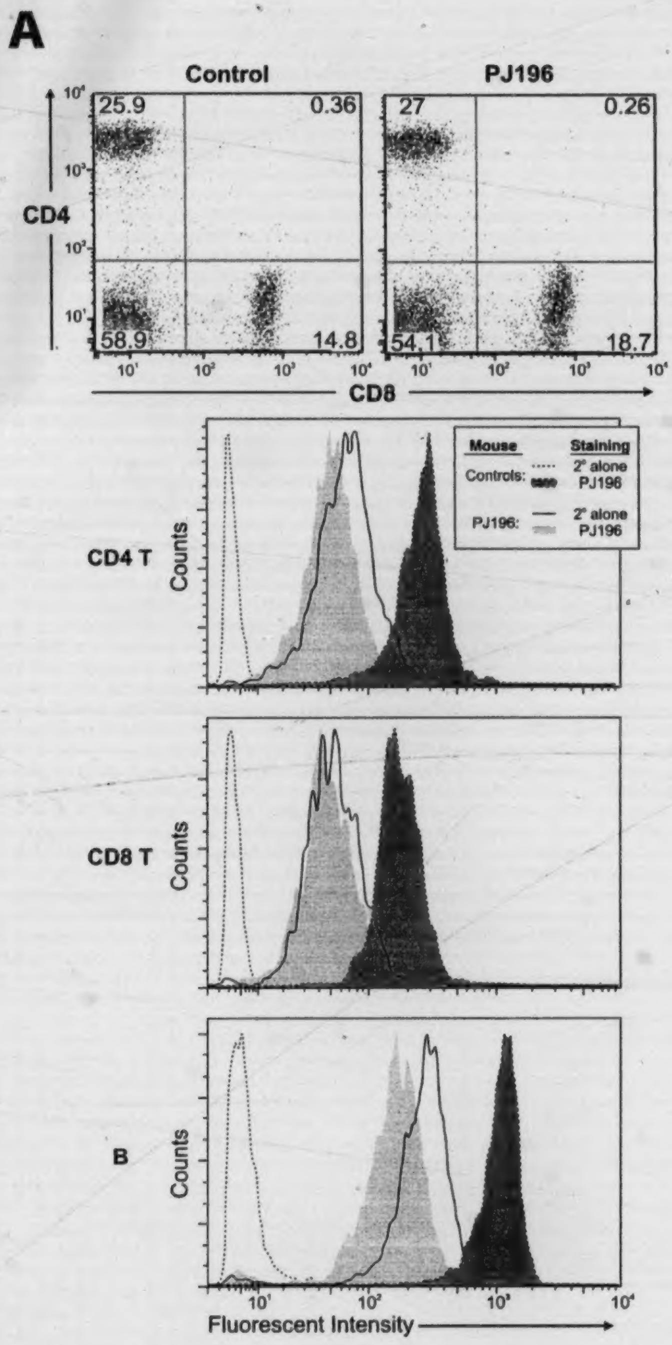
A
Control
PJ196
CD4
CD8
25.9
0.36
58.9
14.8
27
0.26
54.1
18.7
10¹
10²
10³
10⁴
Mouse
Staining
Controls:
2° alone
PJ196
PJ196:
2° alone
PJ196
CD4 T
CD8 T
B
Counts
Fluorescent Intensity

Figure 6-2A

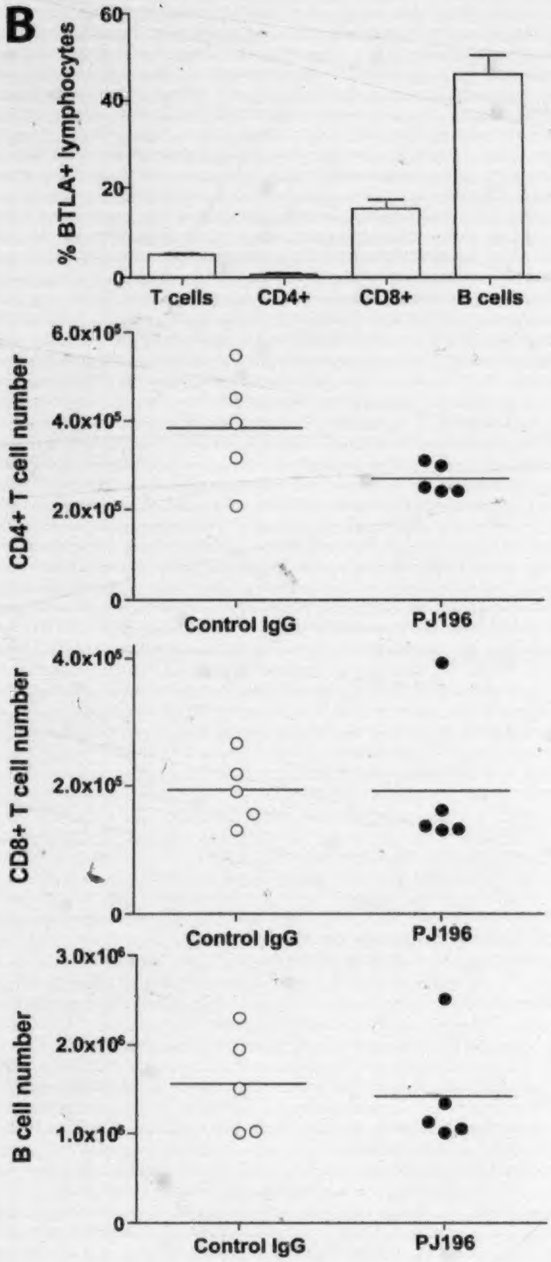
B
% BTLA+ lymphocytes
60
40
20
0
T cells
CD4+
CD8+
B cells
CD4+ T cell number
6.0x10^5
4.0x10^5
2.0x10^5
0
Control IgG
PJ196
CD8+ T cell number
4.0x10^5
2.0x10^5
0
Control IgG
PJ196
B cell number
3.0x10^6
2.0x10^6
1.0x10^6
0
Control IgG
PJ196

Figure 6-2B

Figure 6-2 Anti-BTLA mAb PJ196 decreases the surface expression of BTLA, but does not cause depletion of on B and T lymphocytes

A. After *in vivo* administration of two doses of 0.1mg anti-BTLA mAb the relative proportions of CD4+ and CD8+ T cells did change. In control mice, the splenocytes incubated with a fluorescently-labeled secondary Ab did not stain; however, if the cells are stained with anti-BTLA mAb PJ196 plus secondary Ab the B cells stain with the highest fluorescent intensity followed by CD4+ and then CD8+ T cells. In mice treated with anti-BTLA mAb, the fluorescent intensity as detected by secondary Ab staining is significantly decreased. In addition, the this *in vivo* dose was not completely saturating as the intensity increased with the addition of PJ196 *in vitro* plus secondary Ab. **B.** C57BL/6 splenocytes were transferred to a B6-RAG-KO recipient for lymphocyte characterization in the absence of contaminating thymic and bone marrow emigrants. The BTLA expression was highest on B cells. After treatment with anti-BTLA mAb PJ196 there was no detectable decrease in absolute B, CD4+, or CD8+ T cell numbers. Analysis of two independent groups was performed using a Student's t-test, and a one-way analysis of variance (ANOVA) was used to analyze multiple groups. A p-value <0.05 was considered significant. All staining antibodies were purchased from eBioscience (San Diego, CA, USA) and staining was performed according to manufactures recommendations. Stained cells were detected with a FACSCalibur flow cytometer, and analyzed using CellQuest software (Becton-Dickson, Braintree, MA, USA).

6.3.3 Anti-BTLA mAb PJ196 and CTLA4Ig causes accumulation of regulatory T lymphocytes within the islet allograft

The decreased BTLA expression could potentially be due to depletion of BTLA expressing cells. To test this possibility, we followed lymphocyte number and coinhibitory receptor expression after adoptive transfer of approximately 30 million C57BL/6 splenocytes into C57BL/6-RAG-KO (B6.129S7-*Rag1*^{tm1mom}/J) recipients, without new thymic or bone marrow emigrants. The recipients were subsequently treated with anti-BTLA mAb PJ196 or IgG1 isotype control at 0.50mg on day 0 and 0.25mg on days 2 and 4. Anti-BTLA mAb PJ196 treatment did not change the absolute lymphocyte numbers compared to control with no evidence of depletion. Over 50% of B cells in C57BL/6 mice splenocytes express BTLA after transfer; however, anti-BTLA mAb PJ196 did not cause any significant reduction in absolute B cell, CD4+, or CD8+ T cell numbers, and did not cause lymphocyte depletion (FIGURE 6-2B).

Long-term engrafted islets were harvested and fixed in formalin before paraffin sectioning, and were stained for insulin using immunoperoxidase, and with hematoxylin & eosin, as previously described (*21*). A minority of grafts from CTLA4Ig-treated mice achieved long term graft survival and demonstrated positive insulin staining with a small amount of lymphocyte infiltration (FIGURE 6-3A). In contrast, staining demonstrated functioning islets positive for insulin in allografts from mice treated with anti-BTLA mAb PJ196-treated mice despite marked cellular infiltration with or without CTLA4Ig. The infiltrates further suggest a non-depleting mechanism of action for PJ196.

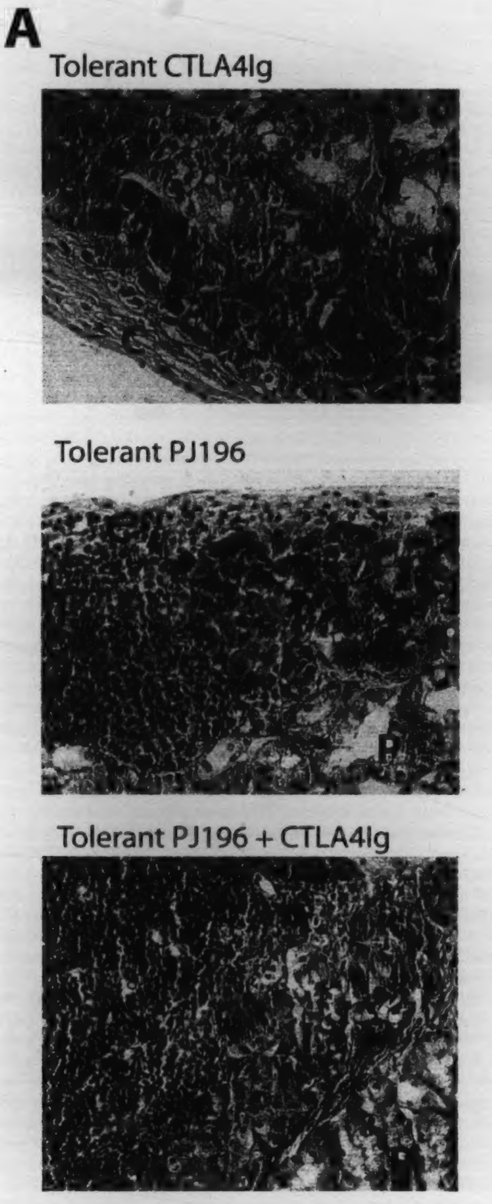
A
Tolerant CTLA4Ig
Tolerant PJ196
P
Tolerant PJ196 + CTLA4Ig

Figure 6-3A

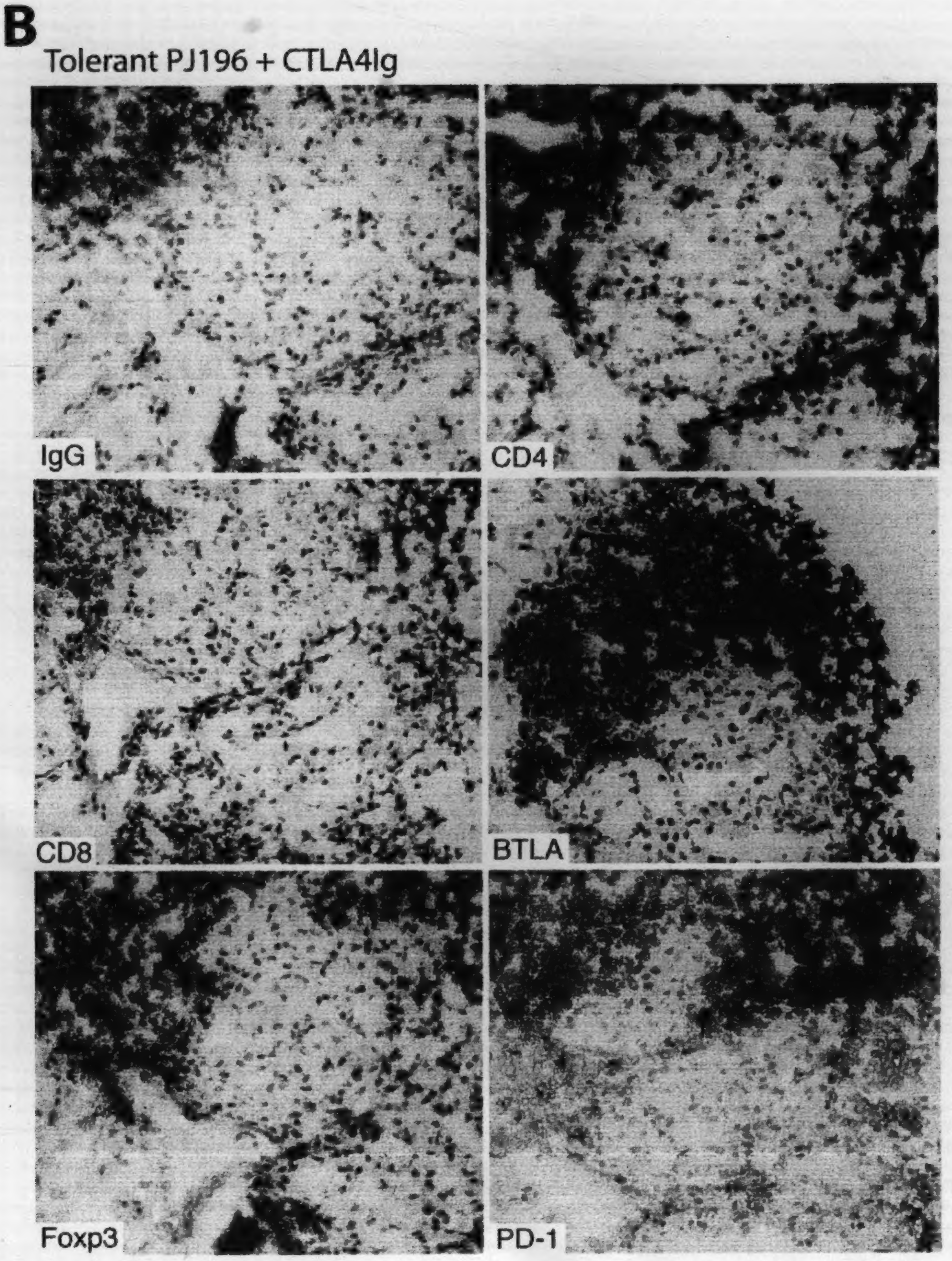
B
Tolerant PJ196 + CTLA4Ig
IgG
CD4
CD8
BTLA
Foxp3
PD-1

Figure 6-3B

Figure 6-3 Anti-BTLA mAb PJ196 and CTLA4Ig prevents islet graft destruction despite massive cellular infiltration, which consists mainly of CD4+Foxp3+PD-1+ lymphocytes
A. A representative islet cross-section for grafts recovered from long-term engrafted mice treated with CTLA4Ig alone show intact islets with positive insulin (brown) staining and very little cellular infiltrates. By contrast, mice treated with anti-BTLA mAb with or without CTLA4Ig demonstrate marked cellular infiltrate despite functioning islets (The renal capsule is labeled **C**, the islet is labeled **I**, and the renal parenchyma is labeled **P**. All sections were stained with insulin, and hematoxylin and eosin, x400). **B.** Immunoperoxidase staining of anti-BTLA mAb- and CTLA4Ig-treated tolerant grafts showed BTLA+ cells isolated in the periphery, with little CD8+ cells scattered throughout, while large numbers of CD4+Foxp3+PD-1+ lymphocytes surround the functioning islets.

To characterize the cellular infiltrates, cryostat sections were stained using mAbs (eBioscience) to murine CD4, CD8, BTLA, Foxp3 and PD-1 and an Envision immunoperoxidase kit (Dako) (*22*). Anti-BTLA mAb have also been implicated in prolonging median survival time by increasing PD-1 expression in fully MHC-mismatched cardiac transplantation model (*15*), and recent evidence suggests local regulatory T (Treg) cell activity at the site of a transplanted graft may at least in part be important for suppressing T cell activity and promoting graft tolerance (*23, 24*). The cellular infiltration was mainly distributed around the edges of the functioning islet allografts in mice treated with combined anti-BTLA mAb PJ196 and CTLA4Ig (FIGURE 6-3B). BTLA+ lymphocytes dominate the periphery, and a small number of CD8+ cells were scattered throughout. However, CD4+, Foxp3+, and PD-1+ cells were found to surround the islet perimeter, and may function as regulatory cells to prevent graft rejection.

6.4 DISCUSSION

In summary, while CTLA4Ig may reduce initial T cell expansion, but with multiple possibly redundant co-signaling pathways it was marginally effective in promoting indefinite islet allograft survival when used alone. Unexpectedly, anti-BTLA mAb PJ196 also significantly prolonged graft survival over controls, and the addition of CTLA4Ig potentiated the effects of anti-BTLA mAb. We demonstrate that anti-BTLA mAb PJ196 does not deplete, but rather causes down-regulation of surface BTLA; and, we speculate that combined treatment enhances the environment for development of Tregs to prevent graft rejection, as demonstrated by histological findings. Amongst a number of mechanistic possibilities, we favor the possibility that PJ196 may downregulate coinhibitory signals on regulatory T cells, enhancing their function. Alternatively, PJ196 may have an agonistic function on BTLA expressing effector cells *in vivo*. Since targeting the receptor with a mAb in the absence of depletion, can promote graft survival and is enhanced in the context of costimulatory blockade, the BTLA negative co-signaling pathway may provide another novel adjunctive approach to transplantation tolerance in the future.

6.5 ACKNOWLEDGEMENTS

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CHAPTER SEVEN

THE TIM FAMILY OF CO-SIGNALING RECEPTORS: EMERGING TARGETS FOR THE REGULATION OF AUTOIMMUNE DISEASE AND TRANSPLANTATION TOLERANCE

NOTE: A previous version of this chapter was
accepted for publication and is currently in press in
Cell Transplantation, and the authors on this paper include:
Truong W, and Shapiro AMJ.

7.1 OVERVIEW

Currently, lifelong immune suppression regimens are required for solid organ and cellular transplantation and carry significant increased risk of infection, malignancy, and toxicity. For non-life-saving procedures such as Islet transplantation, the risk-benefit ratio of lifelong immunosuppression versus benefit from transplantation requires even more careful balance. The search for specific agents to modulate the immune system without chronic immunosuppression is important for the broad application of islet transplantation. The T cell immunoglobulin mucin (Tim) family is a distinct group of co-receptors that are differentially expressed on T_H1 and T_H2 cells, and have the potential to regulate both cytotoxic and humoral immune responses. Completed murine studies demonstrate Tim pathways may be important in the regulation of tolerance to self (auto-), harmless (allergic), and transplant (allo-) antigen; however, the potential impact of targeting Tim co-receptors has yet to be fully explored in transplantation tolerance induction or autoimmune disease. The current review examines the impact of Tim co-receptor targeting as an emerging therapeutic option for regulating autoimmune diseases and prevention of allograft rejection.

7.2 INTRODUCTION

Cellular and solid organ transplantation requires lifelong immunosuppression, often associated with a myriad of side effects. These side effects (1, 2) may be harder to justify in the setting of non-life-saving transplants such as islet transplantation in type 1 diabetes mellitus (T1DM) (3). The procedure still requires lifelong immunosuppression to prevent both allograft rejection, and recurrent autoimmune diabetes (4, 5). Helper T cells play a pivotal role in both autoimmune disease (6, 7) and transplantation rejection (8). The activation of naïve CD4+ T helper (T_H) cells produces several effector populations including T_H1 and T_H2 (9), T_H3 (10), T_r1 (11), and T_H17 (12), each with distinct cytokine profiles and function. The T_H1 phenotype produces the cytokines interferon-γ (IFN-γ), interleukin-2 (IL-2) and lymphotoxin (LT) and is associated with cell-mediated immune responses directed against intracellular pathogens (13-15). In contrast, T_H2 cells generate the cytokines IL-4, IL-5, IL-6, IL-10 and IL-13, which mediate immune reactions against extracellular pathogens (13, 14). Effector T_H cells require tight control to ensure appropriate response and termination with minimal harm to the host. T_H1 and T_H2 cells produce cytokines which are antagonistic to each other, allowing mutual regulation (16, 17). Understanding the differences between the T_H cell populations provide potential novel therapeutic targets to limit pathogenic and promote tolerogenic responses.

Unrestrained T_H1 activation is implicated in delayed-type hypersensitivity (DTH), rheumatoid arthritis, inflammatory bowel disease (IBD), specific autoimmune diseases including T1DM and multiple sclerosis (MS) (18), and also allograft rejection (19, 20). T_H2 cell activation is critical in the pathogenesis of atopic and allergic diseases such as asthma (14, 21, 22), and has also been linked to tolerance induction (19, 20).

The degree of T cell expansion depends on T cell receptor (TCR) binding affinity (23), co-signaling receptors, and cytokine signals (24). The co-signaling receptors can be positive or negative and the major families include the tumor necrosis factor (TNF) receptor family (25), or immunoglobulin (Ig) superfamily members (26). The T cell immunoglobulin mucin (TIM) proteins represent a novel group of co-receptors that are not only markers for identifying terminally polarized T_H1 and T_H2 cells, but also regulators of the balance between T_H1 and T_H2 activation (27). Currently, the *Tim* gene family includes eight members (*Tim*-1 to 8) on mouse chromosome 11B1.1, and three members (*TIMs 1, 3* and *4*) on human chromosome 5q33.2 (28, 29). The *Tim* gene family is conserved between murine and human species and encodes a type 1 membrane gycoprotein. The Tim proteins share common structures including a signal sequence followed by an immunoglobulin variable region (IgV)-like domain, a highly glycosylated mucin-like domain, a transmembrane region, and a cytoplasmic domain (FIGURE 7-1). In the current review, we highlight the role of Tim family members in the regulation of immune responses, and examine the potential strategies of targeting Tim co-receptors for treatment of autoimmune disease and inducing transplantation tolerance.

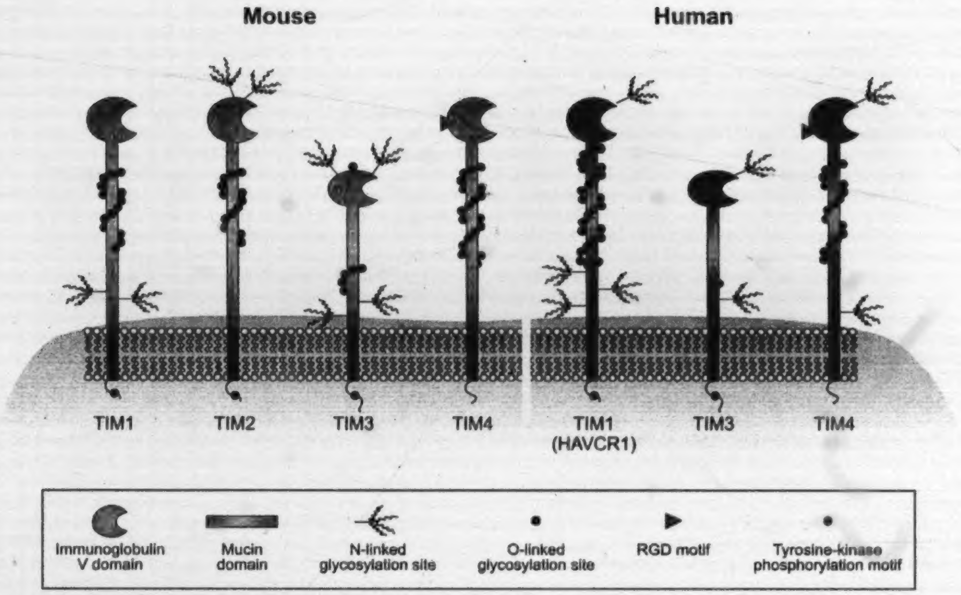
Mouse
Human
TIM1
TIM2
TIM3
TIM4
TIM1
(HAVCR1)
TIM3
TIM4
Immunoglobulin V domain
Mucin domain
N-linked glycosylation site
O-linked glycosylation site
RGD motif
Tyrosine-kinase phosphorylation motif

Figure 7-1 The TIM family
The structure of the TIM family of proteins is conserved from mouse and human. The extracellular portion includes an immunoglobulin variable (IgV)-like domain followed by a mucin-like stalk with various O- and N-glycosylation sites. TIM-4 is unique since it has an RGD (integrin-binding) motif, but no tyrosine phosphorylation motif. HAVCR, hepatitis A virus cellular receptor; TIM, T cell immunoglobulin mucin.

7.3 Tim-1 IS A CO-STIMULATOR OF T$_H$2 RESPONSES

The first *TIM* gene to be discovered, *TIM-1*, was initially identified as a hepatitis A virus (HAV) cellular receptor (*HAVCR*) in the African green monkey in 1996 (30) and subsequently in humans (31). Since HAV infection correlates with protection against the development of asthma in humans (32, 33), this association suggests binding of HAV to TIM-1 may protect against the T$_H$2-dependent immune response and atopy. Furthermore, murine *Tim-1* was identified as an asthma susceptibility gene linked to the T cell and airway-phenotype regulator (*Tapr*) locus (28, 34), which is homologous to the 5q32.2 locus in humans, a region associated with susceptibility to asthma and allergy. *Tim-1* was also detected at high levels in lymph node and kidney tissue (35, 36) and was cloned as kidney injury molecule-1 (*KIM-1*) in rats, after ischemia-reperfusion injury induced expression (37, 38). Rat *KIM-1* has high sequence homology with the human *HAVCR*, and both are considered orthologues of murine *Tim-1* (29).

All recently activated CD4+ T cells express *TIM-1*, which may be involved in directing their differentiation; but after polarization, it is preferentially found on T_H2 cells (36). *Tim-1* is thought to deliver a positive signal to enhance T_H2 responses (FIGURE 7-2A), because the agonistic anti-Tim-1 monoclonal antibody (mAb) (clone 3B3) stimulated T cell proliferation in culture, and promoted T_H2 cytokine production (FIGURE 7-3A) (36). Furthermore, the monovalent F(ab') fragment of the anti-Tim-1 mAb did not stimulate proliferation (36). In an experimental asthma model, *in vivo* administration of agonistic anti-Tim-1 mAb (3B3) prevented tolerance to inhaled antigens by enhancing the T_H2 cytokine response, and increasing pulmonary inflammation, restoring airway hyper-reactivity (AHR) (36). Recent studies suggest allergic inflammatory diseases may be treated by blocking the Tim-1 pathway, since antagonistic anti-Tim-1 mAb (RMT1-10) demonstrated decreased experimental allergic conjunctivitis (EC) by upregulating IFN-γ (39). In another study, blocking anti-Tim-1 mAb (clone 222414) decreased asthma in a murine model by lowering antigen-specific production of the T_H2 cytokines IL-10 and IL-13, while the T_H1 cytokine IFN-γ appeared to be unaffected (40). The role of Tim-1 in autoimmune disease is incompletely understood, and more studies are required in experimental models of autoimmunity, including diabetes.

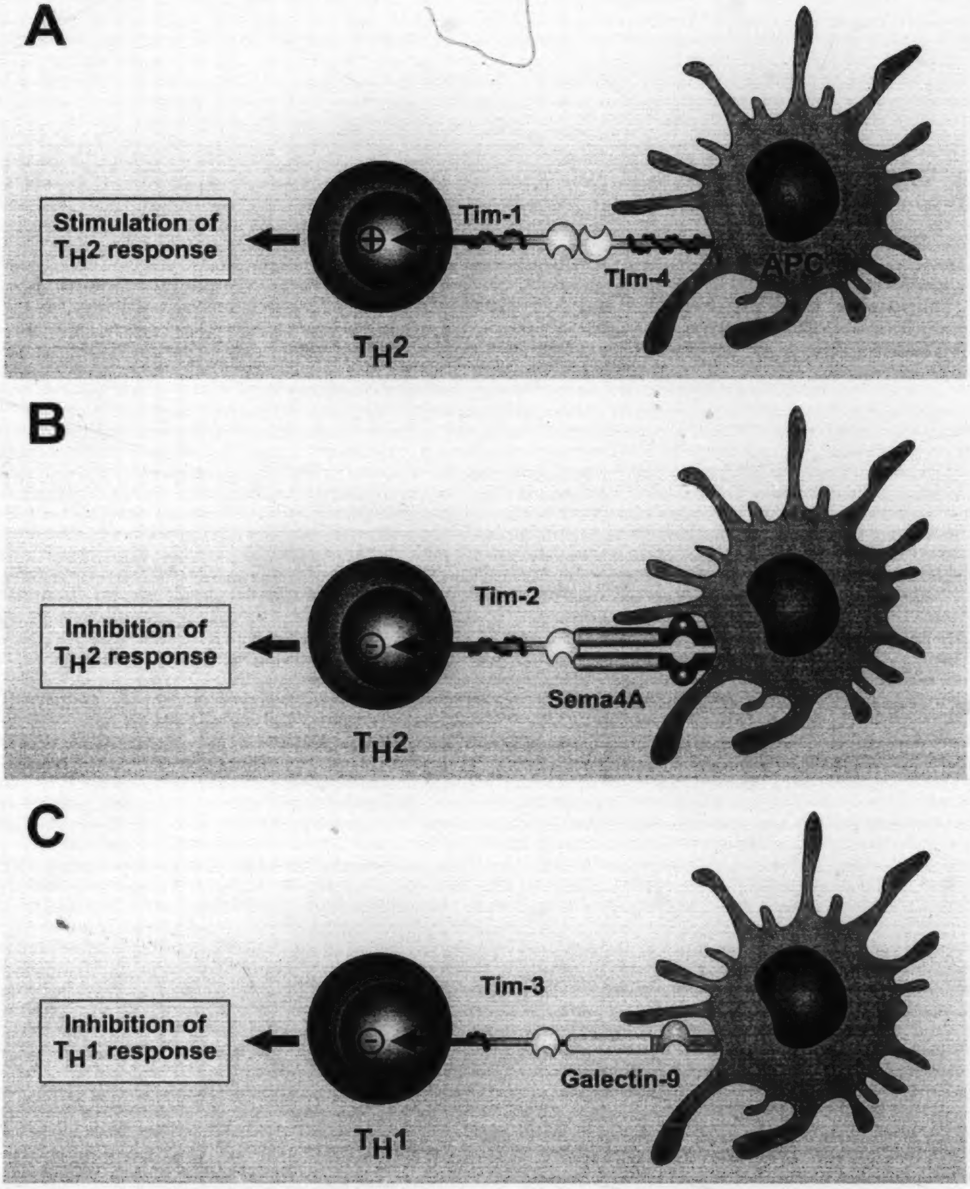
A
Stimulation of T_H2 response
Tim-1
Tim-4
APC
T_H2
B
Inhibition of T_H2 response
Tim-2
Sema4A
T_H2
C
Inhibition of T_H1 response
Tim-3
Galectin-9
T_H1

Figure 7-2 Proposed mechanism of interaction between Tim family co-receptors and their known ligands
(A) Tim-1 is considered a co-stimulator with greater impact on T_H2 cells. Tim-4 is a natural ligand for Tim-1. **(B)** Tim-2 regulates T_H2 response, and likely delivers an inhibitory signal to limit T_H2 cells. **(C)** Tim-3 acts as a co-inhibitory receptor on T_H1 cells.

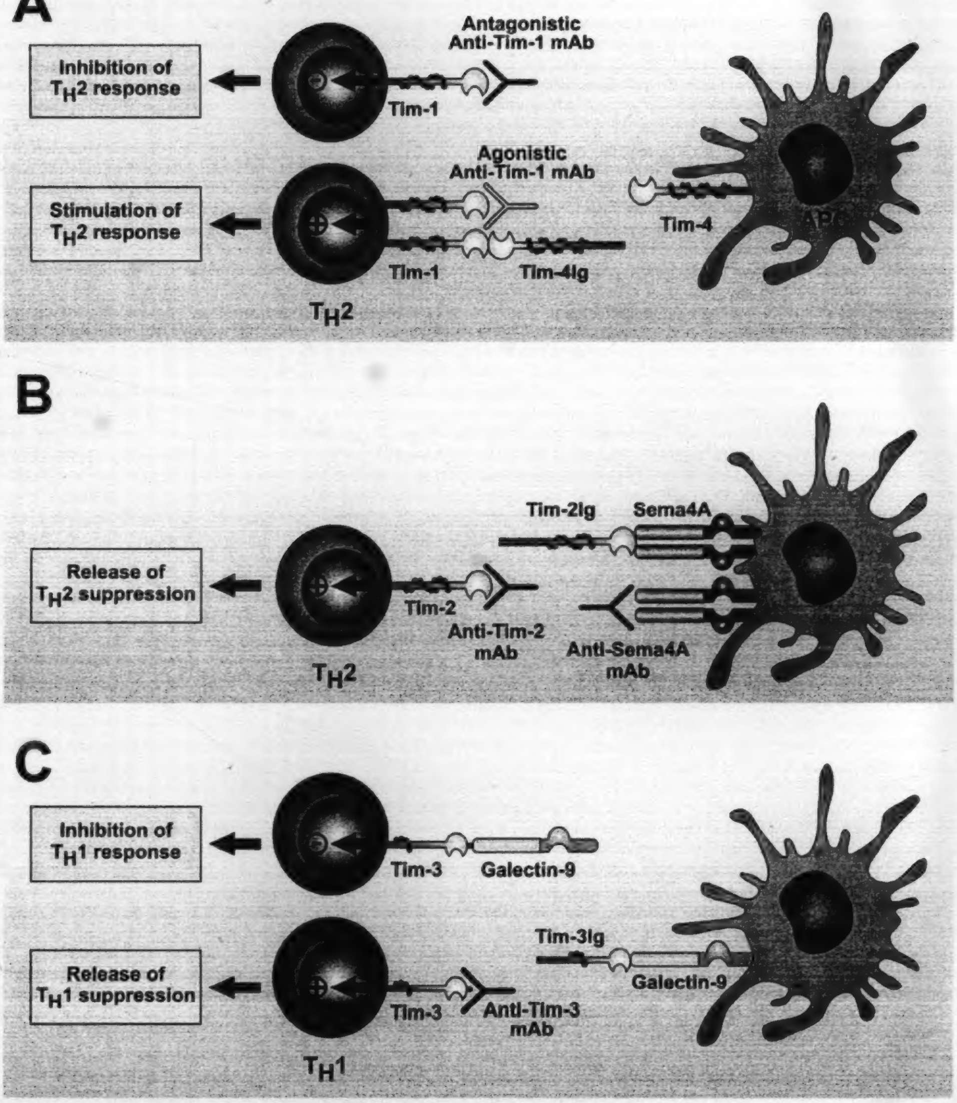
A
Inhibition of T_H2 response
Antagonistic Anti-Tim-1 mAb
Tim-1
Stimulation of T_H2 response
Agonistic Anti-Tim-1 mAb
Tim-1
Tim-4Ig
Tim-4
APC
T_H2
B
Release of T_H2 suppression
Tim-2Ig
Sema4A
Tim-2
Anti-Tim-2 mAb
Anti-Sema4A mAb
T_H2
C
Inhibition of T_H1 response
Tim-3
Galectin-9
Tim-3Ig
Release of T_H1 suppression
Galectin-9
Tim-3
Anti-Tim-3 mAb
T_H1

Figure 7-3. Strategies targeting the Tim family pathways

(A) Both agonistic and antagonistic mAb against Tim-1 have been described. In addition, the Tim-4Ig fusion protein is thought to deliver a positive signal by engagement with Tim-1. **(B)** Current agents include anti-Tim-2 mAb, anti-Sema4 mAb, and Tim-2Ig, which all appear to block the negative signal mediated by Tim-2. **(C)** Tim-3 is a co-inhibitory receptor on T_H1 cells, which can be blocked by anti-Tim-3 mAb and Tim-3Ig. Galectin-9 may deliver a negative signal by engagement with Tim-3.

Ongoing studies emphasize Tim-1 as a general co-stimulation molecule for T cell proliferation. Although, Tim-1 may have a relatively greater impact on T_H2 responses *in vitro*, signaling *in vivo* causes increases in both T_H1 and T_H2 cytokines (36). The first study underway to assess the role of Tim-1 in regulating alloimmune responses used the blocking anti-Tim-1 mAb (RMT1-10) and demonstrated significantly prolonged fully allogeneic cardiac graft survival by co-stimulation blockade and expansion of $CD4^{+}CD25^{+}$ regulatory T (Tregs) cells (41). Furthermore, sub-therapeutic induction doses of rapamycin combined with RMT1-10 resulted in 100% cardiac allograft acceptance (41). Co-stimulatory blockade using anti-Tim-1 mAb should be tested in other allograft models including islet transplantation. Furthermore, the Tim-1 co-stimulatory pathway may impact Tregs, since agonistic anti-Tim-1 mAb (3B3) diminished Treg function, decreased Foxp3 gene expression *in vitro* and *in vivo*, and inhibited new Treg formation, while promoting the differentiation of T_H17 cells (42). Taken together, Tim-1 acts as a co-stimulatory receptor on T cells, with stronger effects on T_H2 than T_H1 cells.

7.4 Tim-4 IS A NATURAL LIGAND FOR Tim-1

Tim-4, a natural ligand for Tim-1 (35) in contrast with other TIM family members, is not expressed on T cells, and is restricted to antigen presenting cells (APCs) with high levels on mature lymphoid dendritic cells (35). In addition, Tim-4 does not have an intracellular tyrosine kinase phosphorylation motif and possesses an RGD motif in its IgV domain, a sequence associated with T cell integrin binding (43). *Tim-4* is detected in spleen and lymph node, and weakly in lung, liver, and thymus (35). *TIM-4* expression in human DCs is increased by Staphylococci enterotoxin B stimulation, which drives CD4+ T cells to differentiate into T_H2 cells (44).

An agonistic Tim-4Ig fusion protein inhibited Treg function (42) and enhanced T cell proliferation mediated by CD3 and CD28 *in vitro* when administered at high concentrations, similar to the effects with agonistic anti-Tim-1 mAb (3B3), but inhibited T cell proliferation at low concentrations (FIGURE 7-3A) (35). One explanation for this disparity may be that TIM-4Ig engages Tim-1 at high concentrations delivering a positive co-signal; however, at lower concentrations may be binding another Tim-4 receptor with higher affinity that has a co-inhibitory action. Alternatively, the activity of Tim-4Ig at different concentrations may also result from Tim-4 binding to a ligand other than Tim-1, since Tim-1Ig can still bind Tim-4$^{-/-}$ cells (35).

7.5 Tim-2 IS A REGULATOR OF T$_H$2 CELLS

Mouse *Tim-2* has high homology with *Tim-1* and is also considered to be an orthologue of human *TIM-1*. Tim-2 is not constitutively expressed on naïve T cells, but is upregulated almost exclusively on T$_H$2 differentiated CD4+ T cells (45). The Tim-2 receptor is also expressed on B cells, and in hepatic and renal epithelial cells (46). One ligand for Tim-2 is Sema4A, a protein found on APCs (47). Another receptor is H-Ferritin, a subunit of ferritin, which buffers reactive oxygen species and induces immunosuppression (48-50).

The role of Tim-2 in autoimmune disease has been explored in experimental autoimmune encephalomyelitis (EAE). The soluble Tim-2Ig (45) or a blocking anti-Sema4A mAb (47) limited the manifestations of EAE. In contrast, the T$_H$2-dependent allergic disease EC was exacerbated by the administration of anti-Tim-2 mAb in the effector phase, but not induction phase of the disease (51). Tim-2$^{-/-}$ mice demonstrate exacerbated lung inflammation in an airway atopic response model and dysregulated expression of T$_H$2 cytokines (TABLE 7-1) (52). Tim-2 also plays a role in allograft tolerance, as the blockade of Tim-2 using Tim-2Ig synergizes with anti-CD154 costimulatory blockade to prevent allograft rejection (27). These studies demonstrate the potential of Tim-2 blockade in treating autoimmune disease and allograft rejection; and therefore, may be useful in preventing disease in the NOD model and prolonging islet allograft survival.

Table 7-1 TIM receptor and ligand deficiency

Model	Strain	Outcome	References
Tim-2$^{-/-}$	Murine	Exacerbated airway atopic disease and dysregulated expression of T_H2 cytokines, with impaired T_H1 response	(52)
Sema4A$^{-/-}$	Murine	Enhanced T_H2 and inhibited T_H1 responses	(53)
Tim-3$^{-/-}$	Murine	Cannot be rendered tolerant with DST and anti-CD154	(54, 55)
	Murine	Resistant to high-dose soluble antigen tolerance induction with increased proliferation and IL-2 production	(56)

Although *Tim-2* and co-stimulatory receptor *Tim-1* are structurally similar, the intracellular tails are significantly different with only 56% sequence identity (53). Current data indicate that Tim-2 acts as a co-inhibitory receptor on T_H2 cells (FIGURE 7-2B) (57). Tim-2 blockade releases T_H2 inhibition and promotes T_H2 cell expansion, possibly facilitating induction or maintenance of peripheral tolerance (FIGURE 7-3B).

7.6 Tim-3 IS A NEGATIVE REGULATOR OF T_H1 CELLS

The Tim-3 receptor was initially described as a marker for T_H1 cells (58). After several cycles of cellular division Tim-3 is significantly upregulated on T cells in conditions that favor the T_H1 phenotype (55, 58), and is associated with IFN-γ production (54, 56). Tim-3 is also found on CD8+ T cells (58), and T_H17 cells (59). Galectin-9, a member of the β-galactoside binding galectin family, was identified as a natural ligand for Tim-3 (60). Galectin-9 is constitutively expressed on naïve T lymphocytes, and is decreased upon activation (60), but maintained or upregulated on CD4+CD25+ Tregs (54).

The role of Tim-3 on T_H1 cells is thought to be inhibitory (FIGURE 7-2C), since administration of galectin-9 severely decreased IFN-γ by inducing T_H1 cell death (60). T_H1 production of IFN-γ increases galectin-9 expression on APCs such as endothelial cells, fibroblasts, and astrocytes, and serves as a natural mechanism to limit the T_H1 response (61, 62). Paradoxically, galectin-9 administration inhibited T_H2-dependent allergic airway inflammation and AHR by preventing the adhesion molecule CD44 from binding with hyaluronan, an interaction that is required for lymphocyte migration into the lung (63). In addition, blockade of Tim-3 pathway consistently induces increased T_H1 responses (FIGURE 7-3C). Treatment with anti-Tim-3 mAb (8H7) or Tim-3Ig significantly accelerated the onset of diabetes in non-obese diabetic (NOD) mice (TABLE 7-2) (54). Anti-Tim-3 mAb also aggravated T_H1-dependent colitis (27), and increased the severity of EAE with massive clonal expansion of macrophages (58). Furthermore, the expression of Tim-3 is significantly lower in patients with multiple sclerosis (MS) despite higher levels of IFN-γ (53). Conversely, in T_H2-dependent allergic airway disease anti-Tim3 mAb (8H7) decreased hyper-reactivity by decreasing T_H2 cells and eosinophils in the lung (64).

Tim-3 signaling is crucial for tolerance induction. High-dose tolerance, induced by administration of aqueous antigen, was abrogated by Tim-3Ig in mice (56). Similarly Tim-$3^{-/-}$ mice were resistant to development of high-dose tolerance, with increased proliferation and IL-2 production compared to wildtype controls (TABLE 7-1) (56). In transplantation, Tim-3Ig prevented the induction of tolerance using donor-specific transfusion (DST) and anti-CD154 (54, 55). Likewise, Tim-$3^{-/-}$ mice cannot be rendered tolerant with the same regimen (TABLE 7-1) (54, 55). A transient upregulation of *Tim-3* occurs in the early stages of anterior chamber-associated immune deviation (ACAID), a form of immune tolerance (65). Anti-Tim3 mAb (RMT3-23 and RMT3-8) treatment in a murine model of acute graft-versus-host disease (aGVHD) accelerated disease with augmented IFN-γ (66). Combined, these data support Tim-3 as a negative co-signaling molecule on aggressive T_H1 mediated auto- and alloimmune responses.

7.7 CONCLUSIONS AND FUTURE DIRECTION

The *TIM* gene family represents a novel therapeutic opportunity, which may impact both autoimmune disease and allograft rejection by shifting the balance between T_H1 and T_H2 cell subsets. The potential use in islet transplantation needs to be investigated. While a few studies are underway to address the role of Tim co-receptors in allograft rejection (27, 41), tolerance (54, 55), and Tregs (42) (TABLE 7-2), none have explored Tim molecule modulation in islet transplantation. Tim co-receptor targeting may be well suited for the prevention of both allograft rejection and autoimmune recurrence in patients with islet transplants for treatment of T1DM.

As the functional roles of Tim co-receptors in the immune response are elucidated, agents targeting Tim pathways may become candidates for drugs that modulate aggressive T cells. Similar to other co-receptor families including TNF family, and Ig superfamily of receptors, *Tim* expression is differentially distributed over time, therefore, the timing of therapy is critical for the desired effect. For example, Tim-1 may act as a general co-stimulatory molecule early after activation since it is expressed on all T cells upon activation; however, after polarization Tim-1 is limited to T_H2 cells. Late Tim-1 targeting will have a greater affect on the T_H2 cell population. Tim-2 or Tim-3 expression on T cells is not constitutive and induced upon activation, which suggests they act late to negatively regulate T_H2 or T_H1 cell responses, respectively.

Tim-2 and Tim-3 proteins have ligands that are identified on APCs, and other lymphocytes. However, ligand expression on tissue may be important for regulating T_H cells and maintaining peripheral tolerance at the site of inflammation or immune response. For example, programmed cell death (PD)-1, a co-inhibitory receptor in the Ig superfamily, has two ligands PD-L1 and PD-L2. PD-L1 on APCs are insufficient to

Table 7-2 Strategies targeting TIM family receptors

Pathway	Reagent/Strategy	Outcome	References
TIM-1/TIM-4 Pathway	Agonistic anti-Tim1 mAb (3B3)	Prevented pulmonary tolerance and promotes airway hyper-reactivity (AHR) in asthma model.	(36)
	Agonistic TIM-4Ig	T cell hyperproliferation & increased cytokine production.	(35)
	Blocking anti-Tim-1 mAb (clone 222414)	Decreased experimental asthma model with lower T_H2 cytokines.	(40)
	Blocking anti-Tim1 mAb (RMT1-10)	Decreased experimental allergic conjunctivitis (EC) & increased IFN-γ	(39)
		Prolonged cardiac allograft survival by proliferation & Tregs.	(41)
		Rapamycin + RMT1-10 resulted in 100% cardiac allograft acceptance.	(41)
	Agonistic anti-Tim-1 mAb (3B3) or Tim-4Ig	Decreased Treg function, Foxp3, and new Tregs, while promoting T_H17 cells.	(42)
TIM-2/Sema4A Pathway	Tim-2Ig or anti-Sema4A mAb	Delayed experimental autoimmune encephalomyelitis (EAE) progression & reduced severity	(45, 47)
	Blocking anti-Tim-2 mAb	Augmented effector phase of EC	(51)
	Tim-2Ig + anti-CD154	Prevent allograft rejection	(27)
Tim-3/ Galectin-9 Pathway	Blocking anti-Tim-3 mAb (8H7) or Tim-3Ig	Accelerates diabetes in non-obese diabetic (NOD) mice	(54)
	Blocking anti-Tim-3 mAb	Aggravates T_H1-dependent colitis	(27)
	Blocking anti-Tim-3 mAb (clone 8B.2C12 or 25F.1D6)	Increased severity of EAE with massive expansion of macrophage	(58)
	Blocking anti-Tim-3 mAb (8H7)	Decreased airway hyperreactivity by lowering T_H2 and eosinophils in the lung	(64)
	Tim-3Ig	Abrogated high-dose aqueous antigen tolerance.	(56)
		Prevents the induction of tolerance using DST and anti-CD154.	(54, 55)
	Anti-Tim3 mAb (RMT3-23 & RMT3-8)	Acceleration of murine acute graft-vs-host disease (aGVHD) with augmented IFNγ	(66)
	Galectin-9	Severely decreased IFN-γ by inducing T_H1 cell death	(60)
		Inhibited allergic airway inflammation and AHR.	(63)

prevent the early onset of diabetes in PD-L1/PD-L2$^{-/-}$ NOD mice, but PD-L1 expression in islets protects against pathogenic self-reactive CD4+ T cell-mediated tissue destruction and effector cytokine production (67). Therefore, the ligands for Tim-2 and Tim-3, sema4A and galectin-9, respectively, should be examined at the site of inflammation and within transplanted tissues to determine if they have a role in maintaining peripheral tolerance.

Tim-2 is expressed on B cells. Similarly B and T lymphocyte attenuator (BTLA), another Ig superfamily member, is found not only on T cells but is expressed at a higher level on B cells. The impact of co-inhibitory receptors, including BTLA and Tim-2, on B cells has not been examined. This may represent a novel approach to targeting B lymphocytes in autoimmune disease and transplant rejection.

One major obstacle for targeting negative co-receptors is the lack of agonistic antibodies that deliver a negative signal *in vivo*. Although not currently available, an agent that engages TIM-3, delivering a negative co-signal to inhibit T_H1 responses, may prevent autoimmune diseases such as MS or T1DM; on the other hand, blocking the TIM-2 signal releasing T_H2 inhibition may be useful in delaying or preventing MS.

In general, Tim family members are structurally similar to an unrelated protein, the mucosal addressin cell-adhesion molecule (MAdCAM)-1, which has distinct ligands, one recognizing the Ig domain and another the mucin domain (68, 69). It follows that Tim family members are expected to have multiple ligands and more complex interactions than initially thought. The complexity is reminiscent of other co-receptors and may prove to be a challenge when attempting to induce tolerance by modulating any one pathway. Targeting multiple co-signaling pathways in T cell activation may be required to prevent auto- and allo-aggressive responses. The Tim family of co-receptors is an exciting and promising novel group of proteins that may have significant impact as adjuncts to the prevention of allograft rejection and recurrent autoimmunity in islet transplantation in the future.

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CHAPTER EIGHT

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTION IN EXPERIMENTAL ISLET TRANSPLANTATION: PROSPECTS FOR ACHIEVING CLINICAL TRANSPLANTATION TOLERANCE

8.1 OVERVIEW

Transplantation has emerged as the accepted therapy for end stage organ failure. New potent immunosuppressive therapy has led to unprecedented short-term graft and patient survival, but has not affected late graft loss. Despite improved glycemic control using a novel glucocorticoid-free regimen in islet transplantation (1), over time there is an inexorable loss of graft function and return to insulin dependence (2). Most immunosuppressive strategies have ubiquitous targets and are non-specific, interfering with effector T cells at a relatively late stage of activation. As a consequence, therapy is administered lifelong to control the immune response after initial lymphocyte expansion, and multiple drug combinations are required to minimize the risks of malignancy, opportunistic infections, and drug-specific toxicity.

Specific immune modulation of novel pathways by redirecting the immune response towards the ultimate goal of tolerance at both priming and effector phases, will be required to advance the field of clinical islet transplantation and to liberate patients from chronic immunosuppression and recurrent disease. The most promising experimental strategies include targeting T cell trafficking receptors and recently identified negative co-signaling molecules. Great enthusiasm surrounds the development of agents that address both allograft rejection and autoimmune recurrence.

Considering these opportunities, the overall goal of this work is to develop tolerance-inducing immune modulating therapies in murine models of autoimmune diabetes and islet transplantation, with potential for rational clinical application. The functional impact of the cell trafficking molecule, FTY720, and the potential role of coinhibitory receptor B and T lymphocyte attenuator (BTLA) in regulating auto- and alloimmunity, have not previously been fully investigated. The major aims of this work included: (i) evaluating the impact of FTY720 exposure on human islets, as a model for testing β cell toxicity for new drugs in islet transplantation; and, (ii) investigating the role of BTLA coinhibitory pathway in autoimmune diabetes and islet allograft rejection and tolerance.

8.2 DISSERTATION, FINDINGS, & IMPLICATIONS

8.2.1 Impact of FTY720 exposure on human islet function

FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol) (3, 4), the first drug in a novel class of S1PR modulators (5, 6), does not inhibit T or B cell activation in secondary lymph nodes, and may preserve host humoral defenses against infections (7, 8). FTY720 down-regulates expression of $S1P_1$ receptor on lymphocytes (9, 10), particularly T cells, thereby sequestering them to secondary lymphoid tissue, which markedly reduces circulating lymphocytes in the spleen, peripheral tissue and transplant grafts (8, 11-14). After cessation of FTY720 treatment, however, lymphocytes may re-express S1PRs and migrate back into the periphery. As a result, FTY720 must be administered continuously for its protective effects.

Oral FTY720 therapy prolongs survival of liver (15-19), kidney (20, 21), cardiac (16, 22-24), and skin allografts (13, 25-27) in various animal models, alone or in combination with cyclosporine (CsA) (13, 16, 20-22, 25, 27), sirolimus (16), tacrolimus (19) and mycophenolate mofetil (MMF) (24). Of particular interest, results from animal models including non-human primates (NHP) show that FTY720 has the potential to be an effective therapy in islet transplantation (28-30).

FTY720 shows promise in treating other autoimmune diseases, preventing or suppressing disease in experimental models of autoimmune myocarditis (EAM) (31), encephalomyelitis (EAE) (32), uveoretinitis (EAU) (33) and systemic lupus erythematous (SLE) (34). It also prolongs host survival in the setting of lethal graft versus host disease

(GVHD) in rats (35). Most importantly, FTY720 in non-obese diabetic (NOD) mice (36) prevents disease onset (37), and even corrects overtly diabetic mice to euglycemia (38).

We examined the potential adverse effects of FTY720 exposure on isolated human islets. We demonstrated that the addition of FTY720 to islets in culture did not significantly impair glucose-stimulated insulin secretion or cell viability, with low rates of apoptosis. We showed that daily FTY720 treatment did not adversely affect human islet function, after transplantation into immunodeficient mice, as determined by random blood glucose, human C-peptide secretion and oral glucose tolerance tests over a 50-day follow-up. Our data support the conclusion that *in vitro*, or *in vivo* FTY720 exposure does not impair human islet function.

8.2.2 Future investigation of S1PR modulators

The sphingolipid S1P promotes vascular and nervous development, and regulates cell trafficking in the periphery and chemotaxis at inflammatory sites by stimulating cell movement (39). The function of its receptor $S1P_1$ expressed on islets is still unclear. Nevertheless, emerging S1PR modulators and other drugs potentially useful in islet transplantation should be evaluated for β cell toxicity using similar techniques before clinical trials.

Although the use of FTY720 itself, is unlikely to proceed in transplantation due to systemic side-effects (40-43), other emerging S1PR modulators are potentially useful in prolonging allograft survival and preventing autoimmune disease, making them well suited for islet transplantation for autoimmune diabetes.

To minimize toxicity, S1PR modulators may be used as induction therapy, when combined with costimulatory blockade or coinhibitory modulation. While T lymphocytes are sequestered to secondary lymphoid tissue by a short course of a S1PR modulator, preventing acute rejection or autoimmune damage, simultaneous co-signaling modulation may induce anergy (44) or regulation (45), while allo-specific depletion strategies (46) may be used to promote long-term tolerance.

Chemokine receptor modulation may be another way to prevent effector lymphocyte trafficking into target tissue (47), and could be tested in combination with other tolerance-inducing regimens. Unlike FTY720, which act as a global chemotaxis inhibitor, modifying the function of at least CCR2, CCR5, CCR7, and CXCR4 (48), chemokine modulation targeting specific T cell subsets may prevent allograft rejection without significantly altering normal immune function and leukocyte surveillance. The multiplicity of chemokine pathways and the lack of available agents to target these molecules hinder progress in this field, but further investigation in this exciting area of combining cell trafficking modulators with co-signaling receptors may result in dramatic impact on tolerance inducing therapy.

8.2.3 The novel BTLA coinhibitory receptor

Evidence supporting the inhibitory role of BTLA is derived from *in vitro* engagement of BTLA by its ligand herpesvirus-entry mediator (HVEM) (49, 50), and *in vivo* (51, 52) from observations of exacerbated disease in EAE (51) and allergic airway inflammation (53) in BTLA-deficient mice.

Based on these studies, BTLA may be important for regulating effector T cells and maintaining peripheral tolerance. Novel tools including specific monoclonal antibodies (mAbs) and fusion proteins have facilitated recent progress in elucidating the roles of co-signaling pathways in T cell priming, activation, differentiation and potentially memory phases. Using available agents, we set out to explore the role of BTLA in auto- and alloimmunity and its interaction with other co-signaling receptors, including PD-1 and CD28 (FIGURE 8-1).

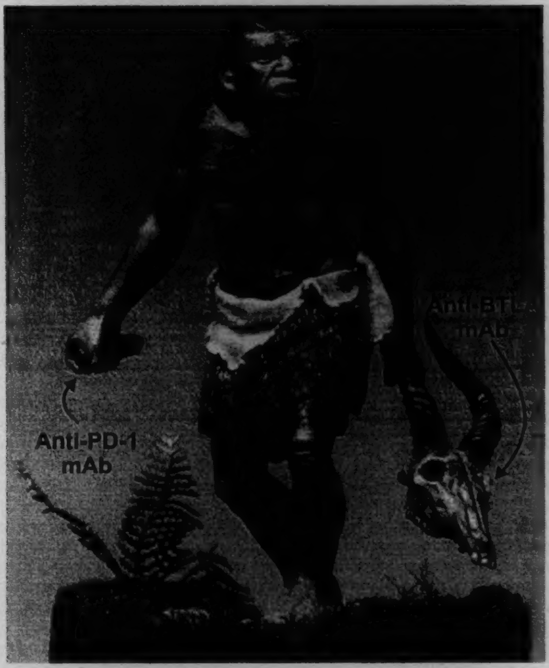


Figure 8-1 Primitive tools

8.2.4 BTLA targeting in autoimmune diabetes development

BTLA is an immunoglobulin (Ig) superfamily member with structural similarities to cytotoxic T lymphocyte-associated antigen-4 (CTLA4) and PD-1. CTLA4 blockade precipitates diabetes only when given within 17 days of birth in susceptible mice (54), regulating early autoimmune destruction of islets. PD-1 blockade induces diabetes in NOD mice regardless of age (55), suggesting involvement in T cell priming and effector phases at the site of immune destruction.

Since BTLA expression is upregulated on activated lymphocytes, its impact may be more pronounced at later stages of T cell activation, regulating at the site of immune response as disease progresses from peri- to invading insulitis. We hypothesized that specific mAbs against the BTLA coinhibitory receptor would accelerate disease in NOD mice, and this may be synergistic with PD-1 blockade. Unexpectedly, we found that anti-

BTLA mAb (clone 6F7) delayed the onset and decreased the incidence of spontaneous diabetes in NOD mice, particularly in older mice and more surprisingly, attenuated anti-PD-1 mAb-induced disease.

8.2.5 *Implications of BTLA targeting in autoimmune diabetes regulation*

We demonstrated that 6F7 selectively depleted autoaggressive CD4+ T cells and B cells capable of antigen presentation, enhancing Foxp3+ regulatory T cells (Tregs) and skewing the cytokine environment. In support of this data, BTLA is lost on terminally polarized T_H2 cells and remains on T_H1 cells, which suggests that as a negative regulator, BTLA serves naturally to suppress T_H1 responses. Depleting BTLA+ cells may be one way to selectively eliminate T_H1 effector cells and shift the balance between T_H1 and T_H2 in favor of generating Tregs, thereby preventing autoimmune diabetes.

We concluded that there is a role for self-reactive BTLA+ T and B cells in autoimmune diabetes progression. Although PD-1 blockade releases autoaggressive lymphocytes from inhibition, 6F7-induced T cell depletion effectively attenuates diabetes precipitated by anti-PD-1 mAb. Therefore, depleting strategies targeting BTLA or therapies enhancing the BTLA negative co-signal may prove valuable in treating autoimmune disease.

8.2.6 *Future investigation of coinhibitory receptor BTLA in autoimmune diabetes*

A recent study showed that T lymphocytes from BTLA$^{-/-}$ mice out-compete wild-type T cells in homeostatic expansion after adoptive transfer to lymphopenic hosts and mice deficient in BTLA have increased numbers of CD8+ memory cells (56). The studies suggest that BTLA negative cells that remain after BTLA+ cell depletion may have a survival advantage. I speculated that the depleting anti-BTLA mAb (6F7) selectively eliminates autoaggressive T cells, and the remaining BTLA negative cells re-populate the niche and shift the balance towards self-tolerance.

To further evaluate the function of BTLA in autoimmune diabetes, investigation in the complete absence of BTLA signaling is required. This can be accomplished using either a purely blocking anti-BTLA mAb (clone 8F4), or development of a NOD-BTLA$^{-/-}$ mouse strain. In a preliminary study, NOD-BTLA$^{-/-}$ mice were generated by crossing C57BL/6-BTLA$^{-/-}$ to NOD mice, which then were back-crossed to the NOD strain for ten generations. Unexpectedly, the NOD-BTLA$^{-/-}$ mice had delayed diabetes development and reduced incidence (20%) compared to wild-type NOD females (80%) at 24 weeks (personal communication with Dr. Kenneth M. Murphy, St. Louis). Mice heterozygous for BTLA also demonstrated delayed diabetes suggesting the results may be complicated by a loss of diabetes-susceptibility genes in the subsequent crosses. Further backcrosses to NOD mice may be required to decrease the background interference.

BTLA targeting should be evaluated for potential protective effects at the time of and after overt disease onset in NOD mice. These studies would enhance our understanding of the stage at which BTLA regulates diabetes. Functional experiments of infiltrating or peripheral Foxp3+ cells may help define a regulatory mechanism after BTLA depletion.

8.2.7 *BTLA in experimental islet allograft survival*

Little is known about the influence of coinhibitory molecules on antigen-specific T cell responses, and there were no completed studies investigating BTLA co-inhibitory pathway in islet allotransplantation. Consistent with previous reports of increased BTLA expression on T lymphocytes after *in vitro* stimulation with PMA plus ionomycin (51), and *in vivo* upregulation of intra-cardiac graft BTLA mRNA expression (57), we observed that BTLA expression increased on splenic T cells upon *in vivo* subcapsular islet transplantation. Therefore, we investigated the role of BTLA in a fully major histocompatibility complex (MHC)-mismatched mouse islet transplant model.

Consistent with its impact on diabetes development in NOD mice we reported that anti-BTLA mAb (6F7) does not accelerate graft rejection. Paradoxically, 6F7, in the context of CTLA4Ig costimulatory blockade, prolonged islet allograft survival indefinitely (>100 d), and induced donor-specific tolerance. We believe the combined therapy induced immunologic tolerance by targeting both early and late phases of T cell activation, specifically CTLA4Ig limited the initial *in vivo* allogeneic T cell proliferation, and 6F7 selectively depleted effector cells in the periphery and increased T cell PD-1 expression, which resulted in an accumulation of Foxp3+ Tregs at the graft site. We concluded that targeting the BTLA pathway in conjunction with CTLA4Ig costimulatory blockade could be a useful strategy for promoting immunological tolerance towards autoimmune diabetes and islet allografts.

8.2.8 Paradoxical role of BTLA

Consistent with inhibitory action of BTLA, partially MHC mismatched allografts, were rapidly rejected by BTLA$^{-/-}$ or HVEM$^{-/-}$ recipients (58); however, not all reported actions of BTLA have been inhibitory. For example, fully MHC mismatched cardiac allografts were rapidly rejected by wild-type mice, but showed slightly prolonged survival in BTLA$^{-/-}$ recipients (58). Additional benefits of BTLA blockade with the clone 6A6 were also seen in allograft recipients treated with a sub-therapeutic course of sirolimus (58). The authors hypothesized that BTLA$^{-/-}$ cells or blocking anti-BTLA mAb in this model upregulated PD-1 and unexpectedly prolonged fully MHC mismatched grafts. We speculate that BTLA signaling may suppress the expression of PD-1; however, the mechanism for PD-1 upregulation after BTLA blockade, or in BTLA$^{-/-}$ mice, has not been completely investigated.

Another example suggesting BTLA may have positive signaling functions under certain conditions is the unexpected finding that BTLA$^{-/-}$ donor T cells failed to sustain GVHD in the non-irradiated parental-into-F1 model, and anti-BTLA mAb (6A6) caused reduced survival of donor cells (59). The authors speculate that a positive signal may be mediated by BTLA signaling or by BTLA acting as a ligand for HVEM. Determining the exact mechanism of this positive BTLA action will require additional studies.

We next tested the hypothesis that a non-depleting anti-BTLA mAb, clone PJ196, will synergize with CTLA4Ig to prolong islet allograft survival. We showed that PJ196 did not deplete BTLA+ cells, but decreased BTLA surface expression and, when combined with CTLA4Ig, caused an accumulation of Tregs at the graft site, promoting long-term islet allograft acceptance. We showed that non-depleting PJ196 prolongs islet allograft survival and suggests that the BTLA pathway is important for regulating peripheral tolerance and may be an effective adjunctive immunomodulatory strategy.

8.2.9 *Future investigation of the BTLA pathway in allograft tolerance*

The BTLA/HVEM pathway represents a novel clinical therapeutic opportunity. Initial studies suggest human BTLA (CD272) may have a similar coinhibitory role. A cell line 293T/BTLA stably expressing the human BTLA protein has been generated and can partially inhibit the proliferation and activation of T cells *in vitro* (60). In addition, the expression of BTLA was increased on pleural fluid T lymphocytes in lung cancer patients. The agonistic human mAb to BTLA (clone 7D7) suppressed T lymphocyte activation and proliferation, and decreased cytokine production *in vitro* (61). Similar to BTLA expression in murine models (62), polymorphisms of BTLA may also exist in humans (63).

BTLA polymorphisms and Ab-specific characteristics must be recognized and further investigation is required before BTLA targeting can be applied in clinic. Another issue that should be addressed before clinical trials is the impact of immunosuppressive drugs on coinhibitory receptor expression. For example, the immunosuppressive drug CsA significantly reduced BTLA expression on CD4+ T cells during activation *in vitro*, while sirolimus had little effect. CsA might preclude the actions of mAbs targeting BTLA and prevent immune tolerance induced by BTLA (64). However, *in vivo* experimental studies are required to further define the role of immunosuppressive drugs on co-signaling receptors.

Since 6F7 has been shown to delay the onset of diabetes and prolong islet allograft survival, the next experimental studies should test whether combined therapy, 6F7 and CTLA4Ig, in NOD recipients could prolong islet allografts. BTLA and other coinhibitory receptors including PD-1, T cell immunoglobulin mucin (Tim)-2, and Tim-3 represent a unique opportunity to enhance natural mechanisms of peripheral tolerance in autoimmune disease and transplant allografts.

8.3 CURRENT STATE OF TRANSPLANTATION TOLERANCE

8.3.1 Immune Tolerance

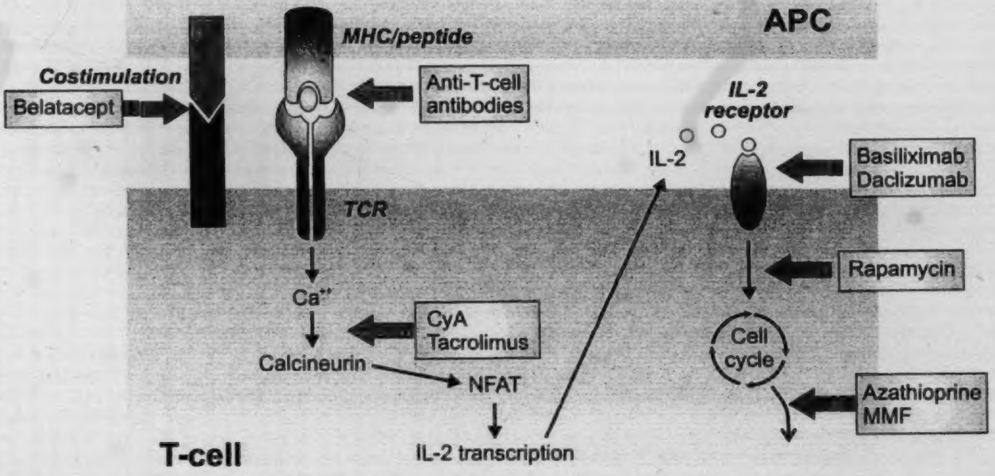
Immune tolerance is the capacity of the immune system to allow the persistence of self and harmless molecules, while maintaining responsiveness to non-self or potentially harmful pathogens. Impaired tolerance is a fundamental defect in many human autoimmune diseases. Our understanding of immune regulation is primarily derived from investigation of aberrant tolerance to self (autoimmune disease), or harmless molecules (allergic disease), in addition to the study of allogeneic transplantation. The objective of preventing specific unwanted immune responses to achieve a steady state of immune tolerance has been the 'holy grail' of transplant immunology ever since the first report of successful experimental transplantation tolerance induction in mice over a half century ago (65). 'True tolerance' implies the absence of acute and chronic rejection, and of donor-specific circulating alloantibody, without immunosuppression (66). The induction of 'true immunological tolerance' in clinic remains elusive.

Although 'true tolerance' is the ultimate goal, I do not believe it is absolutely necessary for advancing the field of clinical transplantation. For successful transplantation, the lack of acute graft rejection, without immunosuppressive drugs, in the presence of immune competence or 'operational tolerance' (67) is what is really important to the patient and clinician. Central tolerance strategies, based on bone marrow transplantation (BMT) to induce mixed chimerism, are robust but involve invasive conditioning regimens which have risk-to-benefit ratios that are currently too high to justify clinically. Peripheral tolerance protocols do not require complex regimens, while still providing antigen specificity. However, the graft must survive for the lifetime of the individual and chronic rejection is an important barrier for any tolerance protocol. Long-term acceptance of MHC-incompatible organ allografts without chronic immunosuppressive therapy has been observed spontaneously in experimental animals, and mainly in liver transplants (68, 69) in humans. Although alloantigen persistence may be one mechanism to maintain peripheral tolerance (70), liver transplants may be more tolerogenic because of the larger initial mass of donor tissue associated with higher number of 'passenger' leukocytes compared to other organ transplants, such as the heart or kidney (71). 'Passenger' leukocytes including donor dendritic cells may migrate to lymphoid tissues and induce apoptosis of alloreactive naïve T cells, and cause antigen-specific activation and subsequent deletion of naïve and effector cells within the liver or thymus (72). I foresee that peripheral induction protocols will require boosts of tolerance promoting donor-specific antigen and "passenger" leukocytes with appropriate immune modulation, not unlike vaccination therapy, but with the opposite goal of decreasing reactivity to a specific antigen.

Ironically, the success of current non-specific immunosuppressive drugs in preventing acute rejection may be an obstacle to testing tolerance-inducing strategies. Currently 'prope tolerance', defined as long-term graft survival with minimal maintenance immunosuppression (73), is achievable in clinical transplantation but I believe any chronic immunosuppression may interfere with tolerance induction and will hinder our ability to detect tolerance, a critical component to evaluating novel tolerance-inducing strategies. Nevertheless 'prope tolerance' is a necessary step towards immunosuppressive-free regimens.

8.3.2 Immunosuppression in clinical transplantation

The majority of current immunosuppressive agents rely on nonspecific cellular depletion or cytokine targeting in T lymphocytes (FIGURE 8-2). Available therapies include: (i) target TCR or interleukin-2 receptor (IL-2R) with polyclonal or monoclonal antibodies (mAbs); (ii) inhibit calcineurin using cyclosporine or tacrolimus, which prevent TCR-dependent production of IL-2 (74, 75); (iii) block cell cycle-specific signal transduction through the IL-2 receptor by sirolimus or everolimus (76-78); and, (iv) block cellular proliferation with anti-metabolites such as azathioprine and MMF, which act through inhibition of purine synthesis (79-82).



APC
MHC/peptide
Costimulation
Belatacept
Anti-T-cell antibodies
IL-2 receptor
IL-2
Basiliximab
Daclizumab
TCR
Rapamycin
Ca''
CyA
Tacrolimus
Calcineurin
NFAT
Cell cycle
Azathioprine
MMF
IL-2 transcription
T-cell

Figure 8-2 Current immunosuppressive drug target non-specific pathways.
MHC (major histocompatibility receptor), TCR (T cell receptor), CyA (cyclosporine A), NFAT (nuclear factor of activated T cells), APC (antigen presenting cell).

Tolerance induction requires some degree of T cell activation. In contrast, most immunosuppressive drugs prevent all T cell response, which results in a temporary ignorant state as long as the therapy is administered, but does not prevent rejection after withdrawal. I believe the first step toward an immunosuppressive-free strategy is to reach a state of 'prope tolerance'. Only certain combinations would be rational, for example, CsA can reduce the expression of the coinhibitory receptor BTLA and strategies specifically targeting BTLA would be ineffective in the presence of CsA. Sirolimus is one notable immunosuppressant with potent action against acute graft rejection, but in animal studies does not prevent the induction of tolerance by several peripheral tolerance strategies (83, 84).

8.4 NATURAL MECHANISMS OF TOLERANCE

Natural immune tolerance occurs by two major mechanisms: central and peripheral pathways. Central tolerance is established at the site of lymphocyte development in the thymus or bone marrow. The majority of self-reactive T lymphocytes are eliminated by intrathymic clonal deletion (85, 86). Central tolerance also eliminates autoreactive B cells in the pre-immune B cell repertoire and can prevent the development of high-affinity autoreactive B cells by the mechanisms of receptor editing (revision) (87, 88), clonal deletion for membrane bound antigens (89-91), and anergy for soluble antigens (92, 93).

Since not all self-antigens are expressed centrally, some self-reactive T cell clones escape into the periphery; therefore, peripheral tolerance mechanisms are required at locations of antigen recognition and presentation in lymphoid tissues. Peripheral tolerance can be achieved by various pathways (FIGURE 8-3) including: (i) deletion of activated T cells; (ii) induction of anergy, an unresponsive state, after delivery of coinhibitory signals or encounter with APCs without costimulation (94, 95); (iii) active regulation of effector T cells; and, (iv) ignorance .

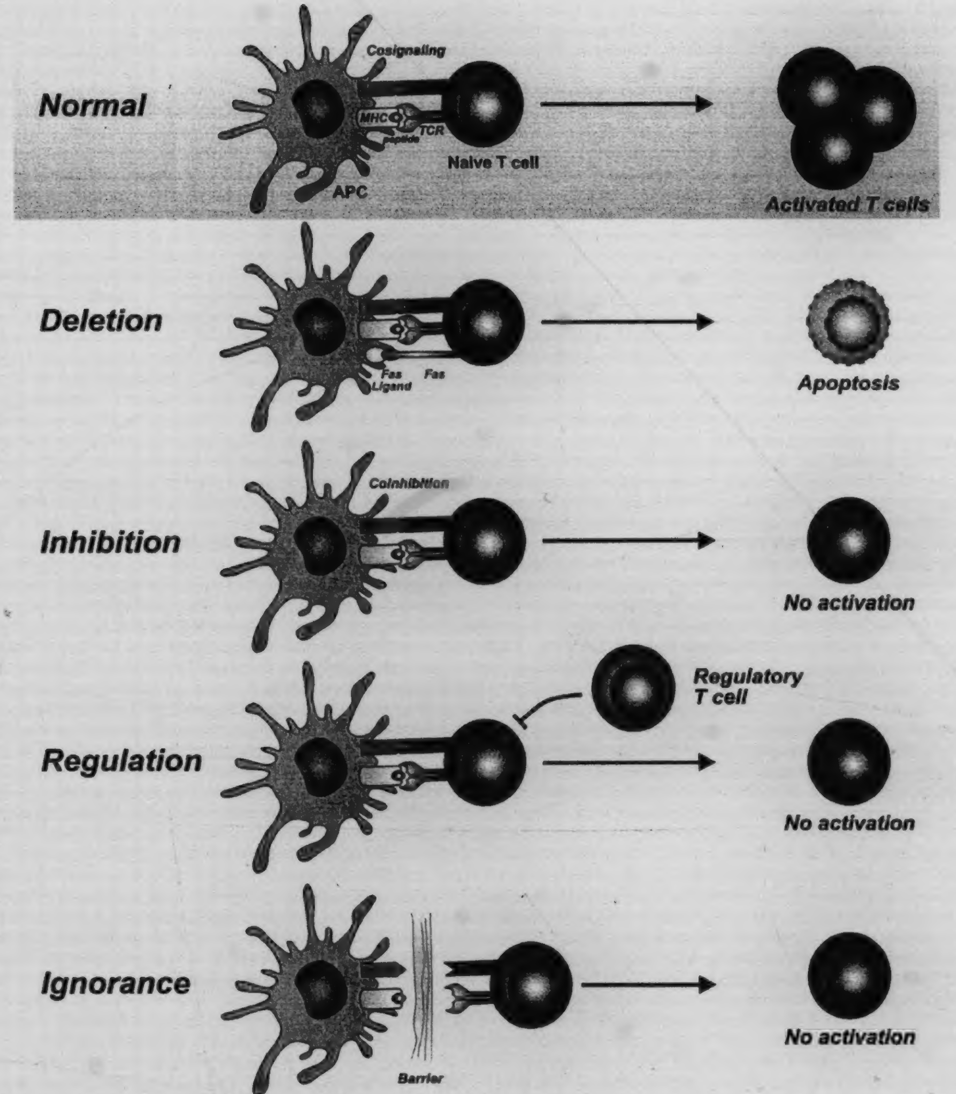

Normal
Cosignaling
MHC
peptide
TCR
Naive T cell
APC
Activated T cells
Deletion
Fas Ligand
Fas
Apoptosis
Inhibition
Coinhibition
No activation
Regulation
Regulatory T cell
No activation
Ignorance
Barrier
No activation

Figure 8-3 Natural mechanisms of peripheral tolerance

8.5 TOLERANCE STRATEGIES

Understanding natural tolerance pathways has led to the development of two main strategies for the induction of transplantation tolerance. Central tolerance strategies involve the establishment of a chimeric immune system so that recipient alloreactive T cells are centrally deleted in the presence of donor APC and antigen in the thymus. Central tolerance mechanisms are robust but inefficient, and would require the central expression of every self-antigen, if it were to operate alone. A separate immune mechanism has evolved to control unwanted T cell activity and maintain tolerance in the periphery. Peripheral tolerance strategies include lymphocyte depletion of alloreactive cells by total lymphoid irradiation or by mono or polyclonal antibodies (46), donor-specific antigen infusion, or costimulatory blockade to induce anergy. Regulation strategies by Tregs, have been reported in murine transplantation models (45), and ignorance has been demonstrated in recipients lacking secondary lymphoid organs (96).

I suspect that a combination of the two strategies will be required for 'true tolerance'. However, both central and peripheral tolerance are not required for 'operational tolerance' in clinical transplantation. Central tolerance strategies based on bone marrow transplantation are invasive, and current protocols for mixed chimerism are not feasible in the clinic. If central strategies are to be effective in clinic, microchimerism and milder conditioning regimens need to be developed. I do not think this will be immediately applicable in the clinic in the near future. Alternatively peripheral tolerance strategies will be important stepping-stones to eliminating the reliance on current immunosuppressive therapy. CTLA4Ig costimulatory blockade may be one way of protecting transplantation grafts from acute rejection without non-specific immunosuppression. The recent clinical trial using Belatacept is a major breakthrough because for the first time an agent that is not a global immunosuppressant can achieve results that are not inferior to the classical immunosuppressive drugs. Therefore this may be a way of getting around the reliance on 'prope tolerance' induction, which is a major barrier to evaluating novel tolerance inducing drugs. As a result, this important advancement will allow more tolerance strategies to be tested in human subjects.

8.5.1 Central tolerance strategies

Bone marrow transplantation (BMT)-based strategies induce a robust form of tolerance mediated by donor derived hematopoietic cells (97). Mixed hematopoietic chimerism following simultaneous BM and solid organ transplant in murine (98) and NHP models induced tolerance (99). In the clinic, tolerance was observed in two patients receiving BMT followed by renal allografts from the same donor (100). Several similar reports of tolerance to organ transplants in patients after donor-specific BMT for hematological malignancy (101-103) suggest that central deletion of donor-reactive T cells can be successful. However, the risk of GVHD has limited attempts to induce mixed chimerism in the clinic.

Less invasive protocols than BMT need to be developed for the translation of mixed hematopoietic chimerism to the clinical setting. In the autoimmune diabetes mouse model, non-myeloablative conditioning achieved mixed hematopoietic chimerism across major MHC barriers. Once mixed chimerism was established, spontaneously diabetic NOD mice accepted donor-specific allogeneic islet grafts and reversed diabetes, despite preserved alloreactive and autoreactive diabetogenic host NOD T cells. In addition, mixed chimerism allowed acceptance of NOD islet grafts, demonstrating reversal of autoimmunity (104).

8.5.2 *Peripheral tolerance strategies*

Peripheral tolerance strategies include lymphocyte depletion, donor antigen infusion, and costimulatory blockade. Depletion using total lymphocyte irradiation resulted in low rates of tolerance induction and high relative risks (105-107), making the technique unsuitable for clinical application. Therefore, antibodies are a more popular strategy for lymphocyte depletion. Anti-CD3 combined with immunotoxin prolonged renal allograft survival in NHPs (108) but grafts showed signs of chronic rejection (109). The addition of 15-deoxyspergualin (DSG), a nuclear factor (NF)-κB inhibitor that suppresses proinflammatory cytokine production and inhibits dendritic cell maturation, improved renal transplants without signs of chronic rejection (110). The anti-CD3 immunotoxin and DSG induced operational tolerance to islet allografts in NHP (111). Another depleting agent anti-CD52 mAb alemtuzumab (campath-1H) is currently being examined in islet transplantation (112). Anti-CD52 mAb depletes T cells, B cells, monocytes, dendritic cells and natural killer cells (113-117). Clinical trials with anti-CD52 mAb in renal transplantation required low-dose maintenance immunosuppression, or 'prope tolerance', to maintain graft function (73, 118). Peripheral depletional strategies still require maintenance immunosuppression, which suggest that combination therapy with other strategies, for example DSG (119, 120), are needed to induce tolerance.

Donor specific transfusion (DST) is another peripheral tolerance strategy (121) that has been shown to be effective in experimental animal models (122, 123). DST combined with anti-CD4 depletion can prolong mouse heart transplants (124, 125). In islet transplantation, DST enhanced graft survival when combined with anti-CD154 costimulation blockade (126). However the technique is unproven in NHPs (127, 128). In addition, the inherent potential risk of sensitization inhibits the progression of DST in pre-clinical trials.

Co-stimulation blockade targeting CD28 and CD154 (CD40L) pathways are the two best-characterized strategies for tolerance induction. Although tolerance induction through CD28 blockade using CTLA4Ig is successful in murine models, it is less effective in NHP. One contributing factor for the lack of success may be due to differences in the B7 receptors between rodent, and NHP or humans. CTLA4Ig has a much lower avidity to its ligand, B7-2, in primates. Nevertheless, CD28 costimulatory blockade using CTLA4Ig (129) or anti-B7 mAb (130, 131) prolongs renal allografts in NHP despite the lack of evidence for tolerance. Manipulation of the CTLA4Ig fusion protein using site-directed mutagenesis to create LEA29Y (Belatacept) (132) has been shown to improve allograft survival in NHP (133). As proof of principle, a recent clinical trial demonstrated that Belatacept was not inferior to cyclosporine A at preventing acute rejection in renal allografts, reducing chronic allograft nephropathy, and maintaining normal Treg populations in the periphery (134). Limitations of the study include the use of low risk patients and no method of assessing tolerance. In theory, targeting CD28 directly using mAbs is better since it does not prevent development of regulatory signals through CTLA4:B7 interactions (135). However, anti-CD28 mAb tends to cross-link its receptor and stimulate T cell activation in human subjects (136). Therefore, careful design of antagonistic anti-CD28 agents is critical for use in clinical trials. An alternative approach to mAbs is the single chain Fv-α-1-antitrypsin fusion protein (ScFv28AT), which binds human CD28 with without causing receptor cross-linking (137).

Anti-CD154 mAb prolonged survival of renal (66) and islet allografts (138) without tolerance induction, because most renal graft recipients developed chronic rejection associated with alloantibodies. The future use of anti-CD154 mAb is uncertain in clinical trials since the observation of serious toxicity leading to thromboembolic events (66, 138, 139). A different anti-CD154 mAb (IDEC 131) combined with sirolimus and donor-specific transfusion have shown some benefit in islet transplantation in NHPs (140, 141) but all were eventually rejected. As an alternative to targeting CD154 in NHP, the chimeric Ab targeting CD40 (Chi220, BMS-224819) has shown promise in renal allografts (142), and in islet transplantation when combined with LEA29Y (143). Blockade of other costimulatory receptors including inducible costimulator (ICOS) (144) has been tested in murine models of islet transplantation but further testing in NHPs is required before clinical trials.

Current co-stimulatory blockade strategies suppress the immune system rather than induce tolerance in large animal and human subjects. Emerging co-inhibitory modulators may be more efficient in NHP or clinical settings, since they target cells at a later stage of activation. The Ig superfamily members CTLA4 and PD-1 have been implicated in the regulation of peripheral tolerance. In addition, we have tested BTLA as a target for immune modulation. New candidates for peripheral tolerance induction include the Tim family of co-receptors, which are differentially expressed on T_H1 or T_H2 cells, allowing specific T_H subset targeting and adding another level of control in directing the immune response towards tolerance. Tim co-receptors may be similar to PD-1 and BTLA because they function at later phases of T cell activation and are not limited to initial priming like CTLA4.

8.6 CHALLENGES TO CLINICAL TRANSPLANTATION TOLERANCE

Despite successful induction in murine models, tolerance has proved to be elusive in large animal models and in the clinic. The reasons for this discrepancy have not been entirely explored, but the many differences between rodent and human immune systems may be a contributing factor. Prolonged survival is readily achieved in vascularized allografts in murine models, partly owing to the lack of endothelial expression of MHC class II antigens, compared to human recipients (145). Another barrier to tolerance induction is heterologous immunity, which predicts that immunological exposure expands the memory T cell pool of large animals, while inbred strains used for experimental research have a relatively limited pool (146). For example, successful experimental protocols targeting the classical co-stimulatory pathways B7:CD28 and CD40:CD40L, molecules vital in primary T cell activation, are far less effective in preventing allograft loss mediated by memory T cells (147, 148) and CD8+ T cells (149) in humans.

In addition, infection encountered by human subjects may adversely affect tolerance induction, since it may provide a 'danger signal' during antigen encounter (150). Another difference with tolerance induction in clinic relates to the stability of the grafts over time. Many murine models have achieved long-term graft survival without acute rejection, but that does not preclude a state of chronic rejection and should not be confused with tolerance. Graft function may not decline significantly over 100 days in murine models, but deterioration of function may occur over long periods of time (151) both in NHP (152) and in humans (67).

The introduction of new tolerance-inducing protocols faces the ethical issue associated with eliminating immunosuppressive drugs, since the morbidity of a failed transplant is clearly higher than immunosuppressive drug side-effects (153). Furthermore, the routine use of non-specific immunosuppressive drugs may inhibit some mechanisms of tolerance induction. However, new tolerance strategies must be tested, at least initially, with traditional immunosuppressive drugs in clinical trials before moving to completely immunosuppressive-free regimens (154).

The next major barrier to eliminating current immunosuppression is the lack of a reliable assay to diagnose a state of tolerance or detect early rejection. The test for tolerance will likely require multiple complementary assays, considering the many pathways leading to tolerance. Novel approaches to detect tolerance include measuring dendritic cell subset ratios (155), Treg expression patterns (156) and CD8+ cell phenotypes (157), and gene expression profiling using microarrays (158).

Despite limitations the concept of weaning immunosuppression has been described in the clinic. One major series reported the results of 72 kidney, pancreas and liver transplant patients after thymoglobulin induction and tacrolimus maintenance therapy (159). The majority (60%) of transplant recipients were successfully weaned to interval doses of tacrolimus and achieved 'prope tolerance'. However, more remarkably, another study withdrew all immunosuppression in over 60 patients receiving living-donor liver transplants (68, 69). The mechanisms accounting for tolerance induction after withdrawal of immunosuppression are under intense investigation, and may be related to the intrinsic characteristics of liver transplants and lack of brain death-induced donor organ injury. Immune adaptation probably occurs with all surviving grafts, regardless of the type of immunosuppression. Claims that various immunosuppressive drugs can induce tolerance are misleading. Strictly speaking, immunosuppressive drugs may promote tolerance but no drug can induce tolerance, only antigen can induce tolerance (145).

8.7 UNIQUE CHALLENGES FOR CLINICAL ISLET TRANSPLANTATION

Islet grafts are different from solid organ transplants because islets can be cultured, only a small volume of tissue is transplanted, and the procedure is non-life sustaining. For these reasons islet transplantation offers unique opportunities including donor or recipient pre-treatment, a variety of new sites for implantation, and testing of immunosuppressive-free regimens without mortality resulting from graft loss.

Islet transplantation offers treatment for patients with T1DM, however the procedure often requires two or more organ donors to achieve insulin independence and clinical outcomes demonstrate reduced functional islet mass over time (160). Multiple factors contribute to functional islet mass loss including: (i) donor islet injury during brain death (161); (ii) ischemia and anoxia during pancreas preservation and islet isolation (162); (iii) innate immune responses consisting of instant blood mediated inflammatory reaction (IBMIR) (163), activated macrophages (164), and exposure to proinflammatory cytokines; (iv) drug toxicity; (v) metabolic stress; and, (vi) adaptive immunity, both alloimmunity and recurrent autoimmunity.

The detrimental impact of brain death on donor islet yield and function is related to proinflammatory cytokine release (161), and novel therapies directly limiting this damage would improve outcomes. Living-donor islet transplantation may be another strategy to avoid this complication (165).

The adaptive immune response has been recognized as a major detrimental factor in islet transplantation survival (166). However, recent investigations demonstrated that innate immune reactions also have a direct negative impact on islets transplanted into the portal system, and contribute to enhancing adaptive immune responses (167). IBMIR is an inflammatory response with subsequent coagulation and thrombosis. Several emerging potent inhibitors of IBMIR are being developed in experimental models with the intention of direct clinical implementation (168-171).

Islets constitutively express tissue factor (172), which binds clotting factor VIIa, to become a major activator of the extrinsic coagulation pathway leading to thrombin formation and generation of fibrin clots. Recipient pre-treatment using exogenous recombinant murine activated protein C (mAPC), an anticoagulant enzyme (168), downregulated tissue factor expression on islets. This improved glycemic control and stimulated insulin release, after intraportal transplantation in diabetic mice by direct anti-inflammatory and anti-apoptotic effects (169). Furthermore, *in vitro* mAPC directly down-regulated NFκB nuclear translocation in portal vein endothelial cells resulting in decreased expression of the proinflammatory cytokine tumor necrosis factor (TNF)-α (169). Unlike other antithrombotic agents including heparin and tissue plasminogen activator, activated protein C is not associated with significant bleeding risk and may be used in the peri-transplant period to control inflammatory-induced adaptive immune activation.

Islets transplanted into the portal vein are susceptible to ischemia, anoxia, coagulation and inflammatory damage. Human α1-antitrypsin (hATT), the major serum serine-protease inhibitor with activity against many pro-inflammatory and pro-thrombotic molecules (170), has been recently described to exert potent inhibition of pro-inflammatory gene expression and decreased mononuclear leukocyte infiltration (171). Although traditionally used as an anti-inflammatory agent, hAAT monotherapy significantly reduced NK cell, T cell and neutrophil infiltration to protect murine islet allografts from early rejection, until the production of anti-hAAT antibodies (171). AAT has a wide range of islet protective effects that may be very useful not only in promoting engraftment by preventing IBMIR, but also in decreasing allogeneic CD4+ T cell activation by directly downregulating islet MHC class II expression (171).

The intraportal site of transplantation is critical for determining the number of islets required for successful induction of insulin-independence (173). Alternative sites of transplantation including omental pouch (174-177) and small intestine submucosa (178) have been explored in experimental murine models to improve islet engraftment, regeneration, and graft function. However, further evaluation in large animal and NHP models would bring the techniques closer to the clinic.

The islet mass required to achieve insulin independence in T1DM patients is higher than that required for surgically diabetic patients and demonstrates the importance of autoimmunity in graft loss. Furthermore, correcting autoimmune disease in NOD mice using costimulation blockade after autoimmune T cell activation has been disappointing. One remarkable study showed that combined treatment with IL-2 agonist, IL-15 antagonist and sirolimus restored euglycemia in diabetic NOD mice (179).

Another effective agent in preventing auto- and allo-responses is anti-CD3 mAb. Non-activating anti-CD3 mAb given at the time of diabetes onset induced remission in 80% and tolerance in NOD mice (180, 181). In NOD mice treated with anti-CD3 mAb that did not return to euglycemia, syngeneic islet transplants were accepted without autoimmune recurrence. The mechanism of this tolerance has recently been shown to involve transforming growth factor β-dependent Tregs (182). In clinic, the antibody OKT3, a murine mAb, had unacceptable toxicity (183, 184). A humanized anti-CD3 mAb (hOKT3), hOKT3γ1-(Ala-Ala) was developed consisting of the murine OKT3 binding region, with modifications to the human IgG1 Fc portion. Clinical trials using the modified antibody hOKT3 demonstrated an acceptable side-effect profile in renal allograft recipients (185) and psoriatic arthritis patients (186). A phase I/II randomized controlled trial demonstrated short-course treatment with hOKT3 prevented progression of insulin loss (187), improved C-peptide responses to mixed meal challenge, reduced HbA_{1C} over 2 years in new onset T1DM (188). This is an attractive agent for islet transplantation not only because of benefits for patients with T1DM (189), but is also the potential prolongation of islet allograft survival. The clinical study using hOKT3 induction and low dose sirolimus and tacrolimus immunosuppression, as part of a single donor islet transplantation trial, reported four of six patients achieving insulin independence with normal HbA_{1C} levels, with no hypoglycemic events, for over 1 year (190). Treatment with hOKT3 is associated with increased CD8+ T cells, peripheral Tregs and serum IL-10 production, a T_H2 cytokine (190). Although remarkable this is a preliminary study conducted using recipients with ideal donors factors. The next steps would require evaluating the effectiveness of therapy in patients with less stringent selection criteria.

Lessons learned from islet transplantation may have far-reaching implications for all types of transplanted tissues. Islet transplantation is a unique opportunity for testing tolerance strategies, where the failure of the graft does not carry the same risk as with life-sustaining solid organ transplants.

I believe the importance of innate immune responses in triggering adaptive responses needs to be addressed. This goal may be achieved through anti-inflammatory drugs as described in this section or by delivery of islets into an alternative site from the portal vein. In addition, novel reagents must prevent acute allo-rejection and recurrent autoimmunity to be effective in islet transplantation.

8.8 IS CLINICAL TRANSPLANTATION TOLERANCE POSSIBLE?

The definition of tolerance in experimental models is different from clinical tolerance. In animal studies, transplantation tolerance can be used to describe the acceptance of a graft without evidence of rejection for a defined period of time, for example > 100 d in the mouse model. In clinical transplant tolerance, the graft must last the life-time of the patient. As a result, tolerance used in the literature may not equate to life long tolerance and is more of an operational term relating to the absence of rejection for a defined period of time. Human subjects are complicated by heterologous immunity relating to memory lymphocytes, and encounter infectious perturbations that may prevent tolerance induction. In addition, donor-specific tolerance can be tested by re-transplantation in animals, but that is difficult to justify in human subjects, and prevents investigators from testing for tolerance in the traditional sense. Therefore, tolerance is seemingly more readily achievable in animal models than in humans, but this is dependent on the expectations and definition of tolerance.

Considering all the barriers, is clinical immunological tolerance possible? Spontaneous tolerance after cessation of standard immunosuppression is the most convincing data to support the possibility of clinical transplantation tolerance. Operationally, the most important tolerance in transplantation is the absence of graft rejection, without continual immune suppression, in the presence of immune competence (67). Although rare, 'operational tolerance' does exist in the clinic today and suggests tolerance is possible under certain conditions, but the actual number of tolerant patients may be underestimated, because some tolerant patients may still be receiving maintenance immunosuppression. 'True tolerance' has, arguably, only been demonstrated in recipients of organ allografts from the same donor of previous BMT. Successful induction of clinical tolerance will require several criteria including: (i) novel tolerance strategies with clinically equivalent acute rejection rates as low as modern immunosuppressive drugs; (ii) robust tolerance using multiple targets to impact immune activation at one or more stages; (iii) stability of graft function over time, including prevention of chronic rejection and perturbations such as infection; (iv) compatibility with current drug therapies; (v) an affordable, reproducible, and timely assay for detecting a tolerant state. Although not all components are in place in the clinical setting at this time, current research promises to deliver many if not all the requirements.

8.9 ALTERNATIVE TISSUE SOURCES

The issue of donor shortage was highlighted in the media when the television production company Endemol in the Netherlands announced a new reality television program called "The Donor Show" (FIGURE 8-4).

de grote
DONOR

Outcry over TV kidney competition

"A Dutch TV station says it will go ahead with a programme in which a terminally ill woman selects one of three patients to receive her kidneys."
—BBC WORLD NEWS 2007/05/29

Figure 8-4 The Donor Show

The proposed scenario for the program was a terminally ill "37-year-old donor, who will make her choice based on the contestants' history, profile and conversation with their family and friends" to determine to whom she will donate her kidneys. The Dutch donor authority has condemned the show, as have kidney specialists in the UK, calling it "totally unacceptable". The ruling political party called for the show to be cancelled, but broadcaster BNN said it would go ahead with the show because it will point out the country's shortage of organ donors.

"The chance for a kidney for the contestants is 33%... This is much higher than that for people on a waiting list. We think that is disastrous, so we are acting in a shocking way to bring attention to this problem. You can have a discussion about if this is distasteful, but finally we have a public debate…"
- BNN Chairman, Laurens Drillich

Before the show was scheduled to air, the creators announced that it was a hoax, and that the show was an attempt to bring the crisis of donor shortage into the public consciousness.

Pressure to develop alternative sources of tissue for transplantation is immense. I believe stem cell therapy may be an answer for the future, but xenotransplantation may be the answer for the near future. Although there are major limitations to xenotransplantation, it is the closest to clinical application and I think it will be an important bridge to advance transplantation before stem cell and future research in alternative organ sources are realized.

8.10 STEM CELL THERAPY FOR β CELL REPLACEMENT

Even if clinical transplant tolerance were readily achievable, the lack of a reliable source of islets would severely limit islet transplantation to a small proportion of patients with T1DM. Stem cell therapy offers a potentially unlimited source of insulin-producing tissue. However, the adult stem cells and embryonic stem cells (ESC) are incompletely characterized, and further studies are required before clinical application.

8.10.1 Pancreatic stem cells

Several potential adult cells have been identified, including pancreatic duct-derived stem cells (191, 192) and nestin-positive islet-derived progenitor cells (193, 194). The feasibility of adult pancreatic ductal stem cell therapy was demonstrated when *in vitro*-generated insulin-producing cells from pancreatic ductal epithelium corrected diabetes in NOD mice (195). However, differentiation protocols have only generated low numbers of insulin producing cells and it is unclear whether the major source of these cells arise from pluripotent ductal stem cells (196) or pre-existing β cells (197). Evidence for *in vitro* generation of insulin-producing cells from nestin-positive islet-derived progenitor cells has been described (198-200) but is still controversial because during early human pancreatic development, endocrine precursors do not express nestin (201, 202). Nevertheless, pancreatic stem cell transplantation may still need to address the problem of recurrent autoimmunity either by immunosuppression or immune modulation therapy.

8.10.2 *Liver stem cells*

Liver and ventral pancreas cells arise from a common stem cell population in the embryonic endoderm during embryogenesis. Transdifferentiation of purified adult rat hepatic oval "stem" cells into pancreatic endocrine cells produced hormones and reversed hyperglycemia in immunodeficient NOD mice (203).

Transduction of the β cell transcription factor pancreas duodenum homeobox-1 (*pdx-1*), which is absolutely required for pancreas development, and the catalytic subunit of human telomerase (hTERT), in human fetal liver cells induced production and physiological glucose-dependent release of insulin. Infusion of these cells corrected diabetes in diabetic immunodeficient mice, but lacked the expression of a number of β cell genes expressed non-β cell genes and had lower insulin content compared to normal β cells (204).

To promote further differentiation of human liver stem cells toward the β cell phenotype, soluble factors including activinA in serum-free media increased the expression of β cell transcription factors and decreased an α cell transcription factor (205). The protocol also decreased the hepatic marker α-1-antitrypsin moving closer to the β cell phenotype. The cells contained human C-peptide and insulin content was increased dramatically, to reach 60% that of normal β cells, and demonstrated physiologic glucose-stimulated insulin release. Furthermore, cells transplanted into immunodeficient diabetic mice achieved stable euglycemia without evidence of cellular replication (205).

Rat liver stem-like epithelial cells reprogrammed with Pdx-1 or its super-active form Pdx1-VP16 fusion protein, trans-differentiated into pancreatic endocrine precursor cells and developed into insulin producing cells to restore euglycemia in diabetic immunodeficient mice without exocrine contamination (206). Recently, use of a lentiviral vector system demonstrated highly effective transduction of hepatic-stem cells with Pdx-1 or Pdx1-VP16, demonstrating similar results (207). These studies show that liver stem cells would be another attractive source for new insulin-producing cells, and developing differentiation protocols using soluble factors may be most feasible.

8.10.3 Hematopoietic stem cells

Bone marrow-derived hematopoietic stem cells (HSC) are an accessible source of adult stem cells, which are able to reconstitute the hematopoietic system (208) and differentiate into ectodermal or endodermal cell lines (209). The capacity of HSC to differentiate into functionally competent pancreatic endocrine β cells (210, 211) is controversial because some of the original studies could not be confirmed (212).

Adult HSCs are unique because they may be able to induce tolerance to auto-antigens and treat autoimmune disease. However, the application of this approach is limited by the risks of GVHD associated with allogeneic HSC transplantation. Syngeneic transplantation would avoid this complication. Transplantation of autologous HSCs modified to express diabetes-resistant MHC class II caused intrathymic deletion of autoreactive T cells and protected NOD mice from the development of insulitis and diabetes (213).

A recent clinical report using autologous hematopoietic stem cell transplantation (AHSCT) in newly diagnosed T1DM patients demonstrated increased β cell function with C-peptide secretion and low levels of HbA_{1c} with minimal insulin or complete insulin independence (214). However, limitations of the study included a lack of control groups, short duration of follow-up complicated by the common ‘honeymoon’ period of relative remission after diabetes onset, and a mechanistic evaluation of tolerance is required (215). Nonetheless, this study is unique because it addresses the important issue of recurrent autoimmunity in stem cell transplantation.

8.10.4 Embryonic stem cells

Embryonic stem cells are an additional source of pluripotent cells derived from the blastocyst, and are able to differentiate into all three embryonic germ layers *in vitro*. Generation of insulin-producing cells from mouse ESCs corrected streptozotocin-induced hyperglycemia in mice after intrasplenic transplantation (216). ESC-derived insulin-secreting cells (217-220) share important features with normal β cells, such as synthesis and processing of insulin and nuclear localization of β cell transcription factors. Human ESC research is at its infancy, and protocols established in murine models are not directly applicable in clinic.

Research in ESCs is surrounded by controversy. Recent investigations suggest that ESC-derived insulin-containing cells do not produce insulin *de novo*, but instead, take up insulin from culture medium after apoptotic cells release insulin (221, 222). Currently, new lines of human ESCs require the destruction of human embryos, a practice which is the subject of pointed legal, ethical and political debate (223-227). Proponents argue that the potential for alleviating human suffering and advancing scientific knowledge is immense; however, opponents of human ESC research value the respect and protection of nascent human life. A consensus for human ESC research is required before stem cell therapy can advance in the field of transplantation or medicine.

8.10.5 *Future of stem cell therapy*

Portal venous or systemic delivery of stem cells faces the same immunological barriers of innate immunity and IBMIR as fully differentiated adult islets. In addition, stem cells from allogeneic donors must still overcome allograft rejection and recurrence of autoimmunity. However, stem cells provide a theoretically unlimited supply of insulin-producing cells for transplantation, and auto-transplantation would avoid alloimmunity. Stem cells represent an ideal target for gene therapy. Stem cell research holds great promise but its potential has yet to be fully realized. I believe many studies are required before the benefits of stem cells may reach the millions of patients suffering from diabetes and other autoimmune conditions.

8.11 XENOTRANSPLANTATION

Although stem cell therapy is promising, viable stem cell-derived tissue replacement will require years of research. The most imminent solution for human donor organ shortage is xenotransplantation. Xenotransplantation offers an unlimited tissue supply with added benefits for transplantation (228), including; (i) no waiting times, for example pig organs are continuously available; (ii) no marginal donors or need for "extended criteria" donors, with optimal organ quality; (iii) no emergency transplantations, making recipient or donor pre-treatment with immune modulation possible, (iv) wider indications for treating more patients and expanding transplantation in countries where cultural limitations exist for deceased organ donation; (v) no need for living donors, discouraging intra-family coercion, and organ commerce and trafficking; (vi) organs would be protected from anti-human leukocyte antigen (HLA) Abs and auto-Abs, eliminating the need for HLA-typing laboratories; (vii) potential for genetic modification and engineering of donor tissue to decrease need for immunosuppressive drugs, for example, inbred pig strains; and, (viii) reducing the cost and easing the work-load of organ procurement teams and organizations.

The major challenges that prevent direct implementation of xenotransplantation in clinical transplantation include: (i) antibody-mediated rejection, either hyperacute rejection (HAR) or acute humoral xenograft rejection (AHXR); (ii) T cell mediated rejection; (iii) potential xenozoonoses transmission to humans, particularly porcine endogenous retrovirus (PERV); and, (iv) public skepticism and ethical issues.

8.11.1 Prevention of acute humoral rejection

The Galα1-3Galβ1-4GlcNAc (α1,3Gal) epitope is expressed on tissues of all mammals except humans and 'old world' non-human primates. Humans and primates have natural antibodies against this epitope. Preformed antibodies mediate HAR, which cause the activation of complement and clotting factors, resulting in vascular endothelium injury and intravascular clotting within minutes to hours of xenogeneic exposure. Depletion of antibodies or complement inhibition can effectively abrogate HAR. Depletion of α1,3Gal-specific natural antibodies from the recipient can be accomplished by using immunoabsorption, injection of soluble glycoconjugates, and by immunoglobulin-specific antibody treatment. Biological reagents causing complement inhibition or depletion include cobra venom factor, plasmaphoresis, and soluble complement receptors.

Various transgenes encoding human complement inhibitory proteins ameliorate damage from HAR. These include CD46, CD55 (decay accelerating factor or DAF), CD59 alone or in various combinations (229). Transgenic pigs expressing the human complement inhibitor (hDAF) have been developed (230). In addition, eliminating the genes encoding α1,3-galactocyltransferase (α1,3-GalT or GGTA1) effectively prevents significant α1,3Gal expression in pig tissue. Use of porcine hDAF-expressing donor organs (231-233) or α1,3-GalT-KO (234-236) organs prevented HAR in NHP but immunosuppression was still required for prolonged function of kidney or heart grafts, and all of these grafts were eventually rejected. AHXR develops over several days or weeks after xenotransplantation, and antibodies to non-α1,3Gal antigens likely mediate the complement-independent process. B cell depletion using anti-CD20-specific mAb does not affect established plasma cells producing antibody and does not eliminate α1,3Gal-specific Abs nor prevent HAR, but may be useful for preventing the development of new antibodies in AHXR (237). Current efforts to address HAR involve multiple combinations of gene knockout and transgenic pig strains, however, transplantation of these tissue may still require exogenous immune modulation to prevent AHXR.

Graft accommodation was described in ABO-incompatible kidney transplants (238, 239), where transplantation grafts were resistant to damage despite the production of anti-donor antibody. The mechanisms of accommodation may include: (i) changes in anti-donor antibody specificity, (ii) changes of donor antigen, (iii) or graft-acquired resistance to injury (240). Acquired resistance may be related to graft upregulation of anti-apoptotic genes (241), or expression of complement regulatory proteins (242). No convincing evidence exists for accommodation in more discordant xenografts; however, research in this area is extremely limited and requires attention.

B cell tolerance has been described in models of mixed xenogeneic chimerism. α1,3-GalT-deficient mice, which produce α1,3-Gal-specific Abs, become tolerant to rat vascularized cardiac xenografts after mixed chimerism is established with α1,3-Gal-expressing xenogeneic bone marrow (243). In addition, mixed xenogeneic chimeras also show tolerance to non-α1,3-Gal antigens.

T-cell independent B cell tolerance has been described in ABO-incompatible heart allotransplantation in infants (244). Factors promoting this type of tolerance may be related to ABO antigen persistence. Since humoral reactions to carbohydrate antigens in ABO-incompatible and xenogeneic organ transplants are similar, understanding the mechanisms of B cell tolerance may be useful in controlling xenotransplant rejection.

Humoral responses play a major role in both xeno- and allo-graft rejection. Therapies that target both T and B cells may have advantages over isolated T cell directed therapy. I speculate that BTLA, which is expressed on both B and T cells, may have significant impact on B cells and therefore on xenogeneic graft survival. While the impact of BTLA on B cells and humoral response requires extensive investigation, we can predict that combining agents, which affect both humoral and cellular immunity will be required for xenotransplantation.

8.11.2 Prevention of cell mediated xenograft rejection

Challenges facing T cell mediated xenograft rejection are similar to those discussed for allograft rejection. Emerging tolerance inducing strategies in xenotransplantation include peripheral and central tolerance strategies.

The combination of conventional immunosuppressive agents required to overcome xenograft rejection represent a huge safety concern because the barrier to xenotransplantation is almost insurmountable compared to allotransplantation. However, with newer approaches including anti-CD40L and costimulation blockade it is now possible to have a xenograft function for more than six months in islet transplantation. Multiple combinations are required to control the vigorous xenogeneic immune response. Costimulation blockade using CTLAIg and CD40L-specific mAb has been promising in rodent xenografts (245), and in porcine to NHPs with maintenance immunosuppression protocols (246). The tolerance induced by β2 integrin inhibitor antilymphocyte function-associated antigen (LFA)-1 with anti-CD40L in concordant islet xenografts from rat to mouse was dependent on the PD-1 coinhibitory pathway (247). This supports the role of coinhibitory molecules in the regulation of xenogeneic as well as allogeneic tolerance, and targeting negative co-signals may be beneficial in this area.

Central tolerance strategies involve invasive techniques such as thymectomy and T cell depletion to induce tolerance. However, mice undergoing this protocol and subsequently receiving porcine thymus grafts accepted donor xenografts, including stringent skin grafts, without immunosuppression (248, 249). Other strategies combining thymus and donor-specific xenografts have also been successful. For example, vascularized composite 'thymokidney' xenografts (250) from pig to baboon show improved survival. Mixed hematopoietic chimerism in xenotransplantation was demonstrated in a pig to mouse model (251). Central tolerance strategies appear to be robust, even in the most stringent models. However, the technical and invasive nature of the procedures must be simplified for clinical application.

8.11.3 Xenozoonoses

Endogenous retroviruses exist in all mammals, and the risk of transmitting infectious agents, for example PERV from pig to human, cannot be eliminated by strict microbial control because these viruses are incorporated into the genome. The endogenous retroviruses do not replicate or cause disease under physiological conditions. However, transmission of PERV could occur after *in vitro* co-culture of pig cells with human cells, and *in vivo* transmission has been observed when immunodeficient mice were injected with porcine cells (252, 253). The possibility of PERV transmission from porcine tissue to humans is a major concern and whether a dormant virus might become activated in a new host and become pathogenic is uncertain (254, 255). Fears of the patient becoming infected, as well as close contacts including relatives and healthcare workers are major concerns, and in a worst case, activated viruses could potentially cause an epidemic. However, in more than 160 porcine tissue recipients as part of pig-to-human transplant trials, no trans-species PERV infection has been reported (256). Nevertheless, future recipients of porcine transplants, and family members of recipients must be carefully monitored for PERV. Informed consent for xenotransplantation with pig tissue must include an understanding that monitoring for PERV may be life-long.

8.11.4 Future of clinical xenogeneic islet transplantation

The first observations of pig cells surviving in the human body was in a pilot study of ten patients receiving fetal porcine islets (257). Islets may be the closest, of all xenogeneic tissue, to clinical application (258), with significant progress in pre-clinical NHP models (246, 259). Four recent studies have reported clinical islet xenograft trials (257, 260-262). One demonstrated C-peptide positivity up to 460 days after fetal porcine islet transplantation in renal allotransplant patients, but no reduction in exogenous insulin requirements. The first study to demonstrate a decrease in exogenous insulin requirement was after intraperitoneal transplantation with encapsulated neonatal porcine islets (260). This study showed positive C-peptide excretion in urine up to 14 months, improved HbA_{1c} over 27 months, and laparoscopic recovery of viable encapsulated islets at 9 years (261). In another study, physical isolation of islet grafts and potential sertoli-cell protection were combined. The transplantation of porcine islets, together with porcine sertoli cells, in steel/Teflon stents, placed subcutaneously in non-immunosuppressed adolescent diabetic patients (262) achieved a reduction in exogenous insulin requirements in half the patients for up to 4 years. The controversial trial was criticized (263, 264) given that the effectiveness of sertoli cells in murine (265-267) or in NHP (267) for immune protection in allotransplantation and xenotransplantation is not well established. Finally, a 2005 report on neonatal porcine islets delivered into the hepatic artery of 20 diabetic patients using steroid-based immunosuppression along with tacrolimus and sirolimus, decreased insulin requirements at 1 year with porcine C-peptide positivity but two patients treated with steroid-free regimen showed no improvement. Most clinical xenograft trials have been based on limited animal models, and although difficult, expensive, and time-consuming, xenotransplantation trials need to be based on promising NHP data that better predict outcome.

Two encouraging NHP studies have been reported using islet xenotransplants. The first study reported long-term survival of adult porcine islets delivered intra-portally to streptozotocin-diabetic cynomolgus macaques, with C-peptide secretion and insulin independence. Basiliximab (anti-CD25, anti-IL-2R) was used for induction, while FTY720 and the sirolimus derivative everolimus for maintenance. By adding human anti-CD154 (AB1793) or leflunomide (a selective pyrimidine synthesis inhibitor), xenograft survival was prolonged even further (268). A second study used total pancreatectomy for diabetes induction in rhesus macaques. Anti-IL-2 receptor and anti-CD154, with maintenance immunosuppression using sirolimus and belatacept (CTLA4-Ig) was successful in prolonging the survival of neonatal porcine islets (246).

8.12 SUMMARY AND CONCLUSIONS

The complexity of the immune system is arguably unparalleled in nature and medicine. The immune response depends on multiple pathways and factors that mediate at different times and levels. It is not reasonable to limit immune modulation therapy to only one specific target or one time point. To maximize effectiveness, tolerance inducing regimens require combined strategies targeting different levels and administered at different times. A large number of molecules have evolved for fine control of independent and coordinated pathways that are overlapping but non-redundant.

Exciting and novel strategies have developed from our understanding of natural tolerance induction including central pathways using BM-based therapy and peripheral mechanisms using depletional or co-receptor modulation therapy. For non-life saving procedures including islet transplantation, the risks of general immunosuppression must be decreased or eliminated for broad application. Current studies have focused on costimulatory blockade to prevent activation signaling. Coinhibitory receptors have only recently been recognized as a major player in limiting the immune response, and targeting this natural mechanism of turning off the immune system may promote tolerance.

Drugs with novel mechanisms of action that are effective for preventing both auto- and alloimmune responses are of highest priority for development in islet transplantation. S1PR modulators and BTLA targeting have the potential to affect responses to self- and alloantigen. We demonstrated that FTY720, a S1PR modulator, does not negatively impact human islet function *in vitro* or *in vivo*. Nevertheless emerging S1PR modulators, or any drug, with the potential to impact islet function should be evaluated for β cell toxicity in an experimental model before clinical application. In addition, we showed that targeting BTLA+ cells could be an effective adjunctive strategy to prevent auto- and allo-aggressive T cell function, while shifting the balance towards an environment more favorable for tolerance.

Although the adaptive immune response is a major obstacle to tolerance, innate immune reactions must be recognized as not only a barrier to initial engraftment, but also as a fundamental activator of adaptive responses. Controlling proinflammatory and prothromobotic molecules may be a potent adjunctive strategy when combined with tolerance induction for transplant allografts.

Over 50 years have elapsed since the first successful attempt to induce experimental tolerance. Currently 'true tolerance' cannot be achieved in clinic on a consistent basis without hematopoietic chimerism. However, risks of GVHD and current protocols preclude its general application. Examples of 'spontaneous', and 'operational' tolerance give hope that it is not impossible. However, clinical islet transplantation may first occur as 'prope' tolerance with minimal immunosuppression before complete elimination of non-specific immune suppression. An efficient method of diagnosing a tolerant state is critical for evaluating novel immune modulating therapy. As tolerance strategies become a clinical reality, the problem of donor shortage must be addressed in the form of an unlimited alternative tissue sources; the most promising are xenotransplantation and stem cell therapy. Given the challenges and remarkable opportunities for advancing autoimmune and transplantation tolerance, the future will be met with enthusiasm and excitement.

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APPENDIX

PUBLICATIONS AND SUBMITTED MANUSCPRIPTS

PUBLICATIONS

1. Truong W, Shapiro AM. Progress in islet transplantation in patients with type 1 diabetes mellitus. Treat Endocrinol 2006;5(3):147-58.

2. Truong W, Shapiro AM. (2007) Islet Transplantation. Shackelford's Surgery of the Alimentary Tract (Yeo CJ, Dempsey DT, Klien AS, Pemberton JH, Peters JH) Saunders Elsevier, Philadelphia, PA. 1422-1430.

3. Truong W, Lakey JR, Ryan EA, Shapiro AM. Clinical islet transplantation at the University of Alberta--the Edmonton experience. Clin Transpl 2005:153-72.

4. Truong W, Hancock WW, Anderson CC, Merani S, Shapiro AM. Coinhibitory T-cell signaling in islet allograft rejection and tolerance. Cell Transplant 2006;15(2):105-19.

5. Truong W, Emamaullee JA, Merani S, Anderson CC, James Shapiro AM. Human islet function is not impaired by the sphingosine-1-phosphate receptor modulator FTY720. Am J Transplant 2007;7(8):2031-8.

6. Truong W, Plester JC, Hancock WW, Kaye J, Murphy TL, Murphy KM, Merani S, Anderson CC, Shapiro AM. Combined coinhibitory and costimulatory modulation with anti-BTLA and CTLA4Ig facilitates tolerance in murine islet allografts. Am J Transplant 2007 (In press)

7. Truong W, Plester JC, Hancock WW, Kaye J, Murphy TL, Murphy KM, Merani S, Anderson CC, Shapiro AM. Negative and positive cosignaling with anti-BTLA (PJ196) and CTLA4Ig prolongs islet allograft survival. Transplantation 2007 (In press)

8. Truong W, Shapiro AM. The TIM family of co-signaling receptors: Emerging targets for the regulation of autoimmune disease and transplantation tolerance. Cell Transplantation 2007 (In press)

SUBMITTED MANUSCRIPTS

9. Truong W, Hancock WW, Plester JC, Merani S, Murphy KM, Anderson CC, Shapiro AM. BTLA targeting attenuates disease induced by PD-1 blockade in non-obese diabetic mice. European Journal of Immunology (2007 submitted)